

**ELECTROPHYSIOLOGY OF RAT ANTERIOR  
PITUITARY RECEPTORS EXPRESSED IN *XENOPUS*  
*LAEVIS* OOCYTES**

by

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This thesis is dedicated to my mother and father.  
I shall always be very grateful for their encouragement and support.

"Better the rudest work that tells a story or records a fact  
than the richest without meaning"  
John Ruskin

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The rest of the work in this thesis is my own.



## SUMMARY

Messenger RNA was isolated from rat anterior pituitary glands and was injected into *Xenopus laevis* oocytes. The anterior pituitary receptors which the mRNA encoded were then expressed into the oocyte membrane.

Expression of anterior pituitary receptors was investigated by intracellular recording and two-electrode voltage-clamp methods. With intracellular recording, membrane depolarisations were induced in oocytes following activation of the expressed receptors for the hypothalamic regulatory peptides, gonadotrophin releasing hormone (GnRH) and thyrotrophin-releasing hormone (TRH). The response to GnRH or a GnRH superactive agonist, buserelin was reversibly blocked by the addition of a GnRH antagonist and that to TRH by the TRH blocker chlordiazepoxide. Control oocytes did not respond to the GnRH analogue or to TRH.

Under voltage clamp activation of exogenous GnRH and TRH receptors was shown to cause a slow, transient inward  $\text{Cl}^-$  current with superimposed current fluctuations which reversed around  $-25\text{mV}$ , compatible with the  $E_{\text{Cl}}$  in *Xenopus* oocytes. The current-voltage relation showed a shift agreeable with the Nernst equation when the external concentration of  $\text{Cl}^-$  was changed and the response was abolished by  $\text{Cl}^-$  channel blockers. Incubation of the oocytes with the G-protein inhibitor pertussis toxin abolished the response to GnRH and TRH. The addition of the calmodulin inhibitor chlordiazepoxide and the blocker of intracellular  $\text{Ca}^{2+}$  release TMB-8 inhibited the response to GnRH and TRH. Thus receptor activation appears to involve a G-protein and the release of intracellular calcium from stores to open  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. A calcium-ionophore (ionomycin) was used to study the calcium stores responsible for the activation of a  $\text{Cl}^-$  current. The response to ionomycin consisted of two phases, which had a differing sensitivity to metabolic inhibitors and to intracellular but not extracellular calcium chelation. The response to ionomycin is shown to mimic the response to receptor activation and appears to be due to the release of two different intracellular  $\text{Ca}^{2+}$  stores. The successful expression of anterior pituitary gland receptors, as measured using two-electrode voltage clamp method, was used to commence the search for the genes encoding the GnRH and TRH receptors with a view to the eventual isolation and cloning of these genes.

Studies of oocytes which had intact follicular layers revealed that the *Xenopus* oocyte has endogenous growth hormone-releasing hormone (GRH) receptors in the follicular layer. Activation of these receptors caused the production of a transient outward  $K^+$  current in a dose-dependent manner, which reversed around -100mV (compatible with  $E_K$  in *Xenopus* oocytes) and was inhibited by the  $K^+$  channel blocker TEA+. The activation of a  $K^+$  current by GRH is shown to be dependent on a rise in cAMP. The possible function of this receptor is discussed.

## SECTION 1

### LITERATURE SURVEY

#### Introduction

The anterior pituitary gland contains many cell types which secrete a variety of protein and polypeptide hormones. The release of these hormones from the anterior pituitary gland is under the control of releasing hormones or factors, which are secreted from axon terminals of small parvocellular neurones of the hypothalamus. These releasing hormones are secreted into a specialised portal system of veins which drain to the anterior pituitary gland. This system is thought to provide a link between the central nervous system (CNS) and the hormonal control of the body.

The main anterior pituitary hormones and the hypothalamic releasing hormones which control their release are:

Growth hormone (GH), the release of which is stimulated by growth hormone-releasing hormone (GRH).

Prolactin (PRL), whose release is stimulated by thyrotropin-releasing hormone (TRH) and inhibited by dopamine.

Adrenocorticotropin (ACTH), the release of which is stimulated by vasopressin and corticotropin-releasing hormone (CRH).

Thyroid-stimulating hormone (TSH) release is stimulated by thyrotropin-releasing hormone (TRH).

Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH), are under the control of gonadotropin-releasing hormone (GnRH).

The present study will concentrate on the GnRH and TRH receptors and their means of eliciting a response.

Gonadotrophin-releasing hormone (GnRH) is a decapeptide which is released in a pulsatile manner from the hypothalamus. It is carried to the anterior pituitary gland by the portal system, where it stimulates gonadotrope cells to release the gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), which regulate gonadal function. From studies with enzyme resistant analogues it became clear that GnRH binds to a specific high affinity membrane receptor on pituitary gonadotrophes (Clayton & Catt, 1981) which as yet has not been cloned or sequenced. GnRH is considered to be important in

the regulation of reproduction, development and steroid dependent tumorigenesis and GnRH agonists have proved useful in the treatment of a variety of endocrine disorders and reproductive organ malignancies (see Clayton, 1987; Filicori & Flamigni, 1988 for review).

Thyrotrophin-releasing hormone (TRH) is a tripeptide synthesized in the hypothalamus and is also transported in the pituitary portal system to the anterior pituitary where it binds to a specific receptor and stimulates thyrotrophin (TSH) release. This, in turn, stimulates the release of triiodothyroxine (T3) and thyroxine (T4) from the thyroid gland, which are responsible for the optimal growth, development, function and maintenance of the body tissues.

It is believed that cloning and sequencing the GnRH and TRH receptors would be beneficial for the understanding the action of GnRH and TRH and also for the development of more effective agonists and antagonists. The cloning of most neurotransmitter and hormone receptors has required the purification of the receptor, the determination of a partial protein sequence and the synthesis of oligonucleotide probes with which to obtain a single clone of a receptor. However, the GnRH and TRH receptors, have not been purified and partial sequences of the receptors have not been obtained. The reason for this lack of success is that, as with neuropeptide receptors (Masu, Nakayama, Tamaki *et al.*, 1987), the GnRH and TRH receptors are present in very small quantities in the cell and are embedded tightly in the plasma membrane. In addition, gonadotrophes represent only 5-15% of anterior pituitary cells.

Therefore an alternative means of obtaining the receptor is needed which allows identification of a functional clone without the need for a prior knowledge of any of its sequence. Such a system has been developed using functional expression of receptors in oocytes of the South African clawed frog (*Xenopus laevis*). By injecting poly adenylated messenger ribonucleic acid (poly (A)<sup>+</sup> mRNA) extracted from a source tissue the expression of the desired receptor can be tested by electrophysiological assay. Activation of some receptors expressed in the oocyte membrane cause the opening of endogenous ion channels. The receptors can then be characterised using standard electrophysiological techniques and a study of the second messenger system is possible.

Should this procedure prove successful, this expression system in oocytes can then provide an assay for the size selection of the particular mRNA encoding the

receptor. This then allows the construction of a size-selected complementary DNA (cDNA) library which should contain the single clone which encodes the receptor. Once the library is constructed, the *Xenopus* oocyte system can be used to screen the library, by testing increasingly smaller fractions until a single clone encoding the desired receptor is isolated. Such a strategy has been used to clone and sequence the mouse brain 5-HT 1c receptor (Lubbert, Hoffman, Snutch *et al.*, 1987), the bovine substance K receptor (Masu, Nakayama, Tamaki *et al.*, 1987) and K<sup>+</sup> channel (Timpe, Schwartz, Tempel *et al.*, 1988).

The structure-function relation of the receptor can also be studied using expression in *Xenopus laevis* oocytes following manipulation of the amino acid sequence by, for example, site directed mutagenesis, where a particular amino acid of the DNA sequence is changed in a region thought to be important for the action of the receptor and the effect on the membrane response is measured. Such a study has been carried out on the sodium channel (Mishina, Tobimatsu, Imoto *et al.*, 1985)

The aim of the present study was to use the *Xenopus* oocyte to express the GnRH and TRH receptors from rat anterior pituitary.

The following section provides a general introduction to a study of receptor expression in *Xenopus* oocytes. It includes literature which has been influential in the preparation of this thesis. It is in 3 parts; the first introduces the morphology, the ion channels present in the plasma membrane and the events which occur upon fertilization of a *Xenopus* oocyte, the second gives a review of the receptors which are endogenous to the oocyte and the third is a review of the exogenous receptors which have already been expressed in *Xenopus* oocyte membranes, the type of response they elicit and the second messenger systems which are involved in the response. In addition, the reasons for the extensive use of *Xenopus* oocytes in receptor expression studies are summarised.

## SECTION 1.1

### CHARACTERISTICS OF THE *XENOPUS* OOCYTE

#### Morphology

In the ovary of a frog, such as *Xenopus laevis*, the female germ cells enter meiosis at the larval stage. Meiosis then stops and the cells start to grow. At this stage the germ cells are called "oocytes" or "immature oocytes" (Masui and Clarke, 1979). Most studies on receptor expression are performed on oocytes

which are fully grown (Stage VI) (Dumont, 1972 and Dumont and Brummett, 1978). At this stage the oocyte is surrounded by the following layers;

(i) the vitelline membrane, an acellular fibrous layer.

3 / (ii) the follicle cell layer. The cells of this layer are connected to the oocyte by follicle cell macrovilli and oocyte microvilli (Wischnitzer, 1966). These contacts form "gap junctions" (Browne, Wiley & Dumont, 1978 and Werner, Miller, Azarnia *et al*, 1985) which allow the passage of certain molecules and ions and provide a direct electrical connection between the oocyte and the follicular cells. These cells also contain receptors for various neurotransmitters and hormones (see later)

(iii) the theca, a layer of connective tissue which contains smooth muscle cells, nerve fibres, oogonia and capillaries (Dumont and Brummet, 1978) and

(iv) a layer of epithelial cells which are a continuation of the ovary wall.

When surrounded by all these layers the oocyte is called a "follicle" or "follicular oocyte".

Inside these surrounding layers *Xenopus* oocytes are spheres 1.2 to 1.3mm in diameter. They are clearly divided into two hemispheres; a brown (animal) hemisphere and a yellow (vegetal) hemisphere, separated by a pale equatorial band. The brown colour of the animal hemisphere is due to the presence of melanin granules in the cortical layer of the cytoplasm (Merriam and Sauterer, 1983).

4 — Most electrophysiological methods are applicable in oocytes. The simplest measurement involves penetration by a single electrode to provide measurement of the membrane potential. The oocytes can easily be penetrated by two microelectrodes, which allows the use of two conventional methods: current-clamp or voltage-clamp. Most studies on oocytes have used the two-electrode voltage-clamp method. Other methods which have also been applied are the vibrating probe to record extracellular currents flowing through the cell membrane at various locations (Robinson, 1979), the patch-clamp method (Hamill, Marty, Neher *et al.*, 1981) to record single channel activity in oocytes in which the follicular and vitelline layers have been removed to expose the plasma membrane, and the big patch for high temporal resolution of macroscopic currents (Leonard, Snutch, Lubbert *et al.*, 1986).



## Properties of the oocyte membrane at rest.

As in the oocytes of other species (reviewed by Hagiwara & Jaffe, 1979; Hagiwara & Miyazaki, 1977), the reported values for the resting membrane potential (RP) in amphibian oocytes are highly variable, ranging between -90 and -20 mV. It has been suggested that the resting membrane potential is not as variable as these results may suggest (Dascal 87). Several factors may introduce errors or discrepancies such as damage caused by electrode penetration (see Hagiwara & Jaffe, 1979 for review) and in denuded oocytes the means of defolliculation (see below)

### (i) The RP in follicle enclosed oocytes

When using electrophysiology, the most reliable estimate of the membrane potential is that obtained with a single electrode when the membrane is allowed to seal around this penetration site for at least 15 min. Under such conditions the resting membrane potential of the *Xenopus* oocyte, when enclosed by its follicular layers is between -40 and -60 mV (Kusano, Miledi & Stinnakre, 1982 and Dascal, Landau & Lass, 1984).

### (ii) The RP in denuded oocytes

Defolliculation, whether manually or by the use of enzymes such as collagenase, causes a shift in the resting membrane potential to more negative values than in follicle enclosed oocytes. In early studies of the cause of this it was concluded that removal of the follicular cells leads to activation of an electrogenic Na-K pump which is not active in the follicle enclosed oocyte (Ziegler & Morrill, 1977; Wallace and Steinhardt, 1977). A later study by Dascal, Landau & Lass (1984) concluded that this phenomenon was due to membrane damage. This damage appears to heal over a period of several hours and after this time the denuded oocytes have a resting membrane potential of between -45 and -65mV.

### (iii) Current voltage characteristics, resistance, and capacitance of the oocyte.

The resting voltage-current characteristic of the *Xenopus* oocyte is linear or close to linear between -100 and -20mV (Kusano, Miledi & Stinnakre, 1982, Dascal, Landau & Lass, 1984). At more positive potentials, an outward rectification is observed that can be partially eliminated by intracellular injection

of the Ca-chelator EGTA (Miledi & Parker 1984). Thus the outward current activated at more positive voltages than -20mV is calcium dependent and is probably carried mostly by Cl<sup>-</sup> (Miledi & Parker, 1984; Barish, 1983; Miledi, 1982). At potentials more negative than -100mV an inward rectification is observed (Kusano, Miledi & Stinnakre, 1982 and Miledi, 1982). Thus the current-voltage relationship can be considered to be linear in the region close to the resting potential (-40 to -60mV).

In fully grown denuded oocytes the  $R_{in}$  is between about 1 and 3 M $\Omega$  (Baud 1983 ; Kado, Marcher & Ozon, 1981). The total capacitance of a fully grown oocyte is about 230nF (Kado, 1983). The specific capacitance of the membrane of the oocyte, calculated by assuming the oocyte to be a perfect sphere, lies between 4 and 7  $\mu$ F/cm<sup>2</sup>, which is several times higher than in most types of cell, where the value is approximately 1  $\mu$ F/cm<sup>2</sup>. These findings confirm the data of morphological studies which show that the surface of the oocyte is significantly increased due to the presence of microvilli and cristae (Kado, Marcher & Ozon, 1981; Kado 1983).

(iv) Oocyte membrane selective permeability and the contribution of different ions to the resting membrane potential.

The membrane of *Xenopus* oocytes, unlike that of toad oocytes (Maeno, 1959), does not behave entirely like a perfect K<sup>+</sup> electrode (Busa & Naticelli, 1985). These findings suggest that permeabilities other than K<sup>+</sup> may partially determine the resting membrane potential. A study of the involvement of sodium by Dascal Landau & Lass (1984), where the external concentration of Na<sup>+</sup> was changed and the Na<sup>+</sup>/K<sup>+</sup> pump was inhibited by ouabain, suggested some involvement of Na<sup>+</sup> in the resting membrane potential, perhaps via a Na<sup>+</sup>-Ca<sup>2+</sup> exchanger. The dependence of the RP on Cl<sup>-</sup> has not been satisfactorily studied. However, as the resting membrane potential is between -40 and -60mV, and the equilibrium potentials for the various ions are : K<sup>+</sup> -100 mV (Barish 1983; Lotan, Dascal, Oron & Lass, 1982), Cl<sup>-</sup>, -25mV (Busa, Fergusson, Joseph *et al.* 1985; Barish 1983; Dascal, Landau & Lass 1984; Dascal & Landau, 1980) and Na<sup>+</sup> +80mV (Baud & Kado, 1984; Soreq 1985), then none of the ions are at equilibrium. Gradients of monovalent cations are maintained by the Na<sup>+</sup>/K<sup>+</sup> pump, but the means of maintaining the Cl<sup>-</sup> is unknown.



## Endogenous ion channels in the membrane of the oocyte.

A large number of ion channels exist in the *Xenopus* oocyte membrane which are activated in a number of ways:

### (A) Membrane currents activated by depolarisation

There is a large diversity of ion channels that give rise to membrane currents on depolarisation. These include  $\text{Ca}^{2+}$  channels (Miledi, 1982; Barish, 1983; Dascal, Snutch, Lubbert *et al.*, 1986), tetrodotoxin sensitive (Parker & Miledi 1987) and insensitive (Baud, Kado & Marcher, 1982)  $\text{Na}^{+}$  channels and  $\text{K}^{+}$  channels which produce a transient (Parker & Miledi 1988) and a slowly inactivating (Parker & Ivorra, 1990)  $\text{K}^{+}$  current.

### (B) Non-specific cation channels

Channels which can be activated by stretching the oocyte membrane have been demonstrated in *Xenopus* oocytes using the patch clamp method (Methfessel, Witzemann, Takahashi *et al.* 1986). Two classes have been distinguished, with different single channel conductances (18 and 25 pS), both seem to be non specific cation channels.

In immature *Xenopus* oocytes, a very slowly developing nonsaturating outward current has been observed upon depolarisation (Baud & Kado, 1984). It appears to involve a non selective cation channel but has not been further characterised.

### (C) Chloride channels

For a study of expression of receptors it will become apparent that the chloride channels of the oocyte are of some importance and therefore require a more detailed discussion.

#### (i) $\text{Ca}^{2+}$ -dependent $\text{Cl}^{-}$ current flowing through the oocyte at rest.

The vibrating extracellular probe method (Robinson, 1979) has shown that a net current flows through the oocyte, entering the animal hemisphere and leaving the vegetal hemisphere, carried by  $\text{Cl}^{-}$  and controlled by  $\text{Ca}^{2+}_i$ . It was suggested by Robinson that this current is due to a passive efflux of chloride over the entire surface of the oocyte, the apparent direction of the current coming from a greater density of  $\text{Cl}^{-}$  channels in the animal hemisphere, which was later confirmed by  $\text{Ca}^{2+}$  injection experiments (Miledi & Parker, 1984).

How the oocyte maintains its electroneutrality with this constant efflux of  $\text{Cl}^-$  ions is still the subject of some debate, the movement of  $\text{Na}^+$  and/or  $\text{K}^+$  have been suggested.

(ii) Voltage-activated  $\text{Ca}^{2+}$  dependent  $\text{Cl}^-$  current.

A novel type of voltage-dependent current in the membrane of the oocyte was described by Miledi (1982) and Barish (1983). Under voltage-clamp conditions when an oocyte is depolarised to a potential more positive than  $-20\text{mV}$  a slow transient current occurs which has an amplitude of  $30\text{-}100\text{nA}$  in physiological solution and a reversal potential of around  $-25$  to  $-30\text{mV}$  and is carried by  $\text{Cl}^-$ . The current is also  $\text{Ca}^{2+}$ - dependent, as the current is suppressed by substitution of external  $\text{Ca}^{2+}$  by  $\text{Mg}^{2+}$  (Barish 1983) and the addition of the calcium channel blockers  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{La}^{3+}$  and  $\text{Ni}^{3+}$  (Barish 1983; Miledi, 1982) and injection of EGTA (Miledi & Parker 1984) and enhanced by increasing the extracellular  $\text{Ca}^{2+}$  concentration (Barish 1983; Miledi, 1982) the calcium being presumed to enter via a voltage-dependent  $\text{Ca}^{2+}$  channel.

In conjunction with these  $\text{Cl}^-$  currents it is appropriate to mention the fertilization events which take place in *Xenopus* eggs

### Fertilization events in *Xenopus* eggs

Upon insemination a fast depolarisation of the membrane potential, called the fertilization potential occurs, which is thought to prevent polyspermy (Cross and Elinson, 1980 and Jaffe, Cross & Picherel, 1983). Such a depolarisation is common in amphibians (Ito, 1972) The above events can also be induced by a number of other, artificial stimuli such as; pricking, injection of the divalent cation  $\text{A23187}$ ,  $\text{Ca}^{2+}$  or the calcium mobilising agent inositol trisphosphate ( $\text{IP}_3$ ) (see Dascal, 1987). The depolarisation caused in this case is called the activation potential. Despite some minor differences these two potentials are very similar (Cross, 1981; Webb & Nuccitelli, 1985).

#### (A) The Fertilization (activation) potential

A wave of depolarization spreads over the oocyte starting at the point of sperm entry or activation (Jaffe, Kado & Muncy, 1985; Kline & Nuccitelli, 1985). This wave precedes a wave of rise in intracellular  $\text{Ca}^{2+}$ , which corresponds with cortical granule exocytosis (Busa & Nuccitelli, 1985). Such a rise in intracellular  $\text{Ca}^{2+}$  is common in amphibians and has been determined in a

number of ways, using the  $\text{Ca}^{2+}$ -sensitive photoprotein aequorin and other  $\text{Ca}^{2+}$ -sensitive dyes or intracellular  $\text{Ca}^{2+}$ -sensitive microelectrodes (Hagiwara & Jaffe, 1979). The source of this rise in intracellular calcium is the subject of some debate, a possible candidate for a  $\text{Ca}^{2+}$  store being cortical endoplasmic reticulum (Gardiner & Grey, 1983; Charbonneau & Grey, 1984). The rise in the intracellular concentration of  $\text{Ca}^{2+}$  induced by fertilization, is from 0.4 to 1.2  $\mu\text{M}$  (Busa and Nuccitelli, 1985). This rise in intracellular calcium was thought to trigger the activation of ionic conductance changes which underlie the fertilization potential. Further study of this mechanism has shown that the activation of phospholipase-C and the production of  $\text{IP}_3$  are involved (Busa, Fergusson, Joseph, 1985).

(B) Ionic conductances that participate in the fertilization potential.

The resting membrane potential of an egg of the frog *Rana pipiens* is about -30mV in a low osmolarity solution (similar to the environment into which a frog egg is released from the ovary). Under such conditions, it has been calculated that the equilibrium potential for  $\text{Cl}^-$  would be about +20mV and that for  $\text{K}^+$  about -160mV (Jaffe & Schlichter, 1985). Therefore opening of  $\text{Cl}^-$  channels will be expected to produce depolarisation. In *Xenopus* eggs the application of sperm causes, after a 30 to 60 second delay, a positive going shift in the membrane potential that reaches a maximum of about +3mV in about 30 sec (Webb & Nuccitelli, 1985). The response consists of several positive going fluctuations, before reaching a steady state. However, potentials as positive as +40 mV were observed in prick-activated eggs (Peres & Mancinelli, 1985). These differences are not yet understood.

It has been known for some years that the current underlying fertilization in amphibian eggs is mostly carried by an efflux of  $\text{Cl}^-$  (Ito, 1972). However, in *Xenopus*, as the reversal potential is not at the equilibrium potential for  $\text{Cl}^-$  (-25mV) (Jaffe & Schlichter, 1985; Peres & Mancinelli, 1985), there would appear to be another ionic conductance activated, this was later shown to be partly due to  $\text{K}^+$  (Jaffe, Kado & Muncy, 1985; Jaffe & Schlichter, 1985) and partly due to a voltage activated  $\text{Na}^+$  conductance.

Thus the main conductance underlying the fertilization (activation potential) is a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel. The activation current is smaller in the vegetal than the animal hemisphere (Jaffe, Kado & Muncy, 1985; Kline & Nuccitelli, 1985). In this respect the  $\text{Cl}^-$  channels of the activation current resemble the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels in the immature oocyte, as mentioned previously. Another similarity between these two channels is that using the patch-clamp

technique the channel conductance would appear to be small, as single channel currents were not seen, but the density of channels appeared to be high (Jaffe, Kado & Muncy, 1985, Dascal, 1987).

Fertilization (or activation) are not the only means of activation of a  $\text{Cl}^-$  current. There are several means of eliciting a  $\text{Cl}^-$  current through the membrane of a *Xenopus* oocyte or egg. Such a current can be induced by:

1. Application of a muscarinic agonist which binds to an endogenous receptor in the oocyte plasma membrane and elicits  $\text{Ca}^{2+}$  release from stores and activates a  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current (see later)
2. Exposure of oocytes injected with brain mRNA which express neurotransmitters receptors such as serotonin (see later).

There are certain similarities between the currents elicited after such manipulations:

1. The  $\text{Cl}^-$  currents evoked by injection of  $\text{Ca}^{2+}$  (Miledi & Parker, 1984) by fertilization (Jaffe, Kado & Muncy, 1985; Kline & Nuccitelli, 1985), or by iontophoresis of ACh (Kusano, Miledi & Stinnakre, 1982) are all asymmetrically distributed, with a higher density in the animal hemisphere.
2. Currents induced by fertilization or by  $\text{Ca}^{2+}$  injection, have been shown to be due to small conductance  $\text{Cl}^-$  channels (Jaffe, Kado & Muncy, 1985), where the  $\text{Cl}^-$  channels could not be detected at the single channel level (Dascal, 1987) and more recently, it has been shown that injection of  $\text{Ca}^{2+}$  or  $\text{IP}_3$  induces the opening of  $\text{Cl}^-$  channels of small conductance (3pS) (Oosawa & Yanagishi, 1989). In addition following injection of rat brain mRNA oocytes expressing glutamate (Oosawa & Yanagishi, 1989) and 5-HT (Takahashi, Neher & Sakmann, 1987) receptors produce membrane responses via endogenous small conductance (3pS) chloride channels.
3. When low concentrations of the  $\text{Ca}^{2+}$  ionophore A23187 or  $\text{Ca}^{2+}$  in the bathing solution cause a rise in the  $[\text{Ca}^{2+}]_i$  there is a significant reduction in both (1) The response to ACh and  $\text{IP}_3$  injection and (2) the  $\text{Cl}^-$  current appearing in response to an increase in  $[\text{Ca}^{2+}]_o$  itself (Dascal, 1987). Which suggest that all these responses have a common process of calcium dependent inactivation

4. The addition of the  $\text{Cl}^-$  channel blocker 9- anthracene-carboxylic acid (9-Ac) inhibits  $\text{Cl}^-$  currents caused by both fertilization and A23187 (Snutch, Leonard, Nargeot *et al.*, 1986; Boton, Gillo, Lass & Dascal, 1987).

On the basis of these findings it may be that one channel type is the carrier of a  $\text{Cl}^-$  current which can be activated in the above mentioned ways and is dependent on the internal concentration of calcium. Hence the channel type would appear to be a  $\text{Ca}^{2+}$ - activated  $\text{Cl}^-$  channel.

The molecular mechanism which underlies the activation of the  $\text{Ca}^{2+}$ - activated  $\text{Cl}^-$  current is, as yet, undecided. Possible explanations include  $\text{Ca}^{2+}$ -dependent phosphorylation, with or without the involvement of the calcium-binding protein calmodulin (Scharff, 1981; Stoclet, 1981), a direct effect of  $\text{Ca}^{2+}$  on the chloride channel, or triggering by  $\text{Ca}^{2+}$  ions of an as yet unknown chain of intracellular events (Dascal 1987).

## SECTION 1.2

### ENDOGENOUS NEUROTRANSMITTER AND HORMONE RECEPTORS

A variety of hormones and neurotransmitters elicit slow membrane current responses in follicle enclosed oocytes. These responses can broadly be divided into two main types.

#### (A) Slow $\text{K}^+$ currents

##### (i) $\beta$ -adrenergic receptors

It was demonstrated by Kusano, Miledi & Stinnakre (1977) that epinephrine evoked a membrane hyperpolarisation in follicle enclosed oocytes. The reversal potential was found to be about -80mV. Since the equilibrium potential for  $\text{K}^+$  is -100mV, it would appear that this outward current is carried mostly by  $\text{K}^+$ , but that another ionic conductance is involved. Pharmacological studies have revealed that the response to epinephrine was blocked by  $\beta$  but not  $\alpha$ -adrenergic blockers, and could be evoked by the  $\beta$ -adrenergic agonist isoproterenol (Kusano, Miledi & Stinnakre, 1982). This suggests that the response was mediated by a  $\beta$ -adrenergic receptor.

### (ii) Purinergic receptors

Lotan, Dascal, Cohen & Lass (1982) demonstrated a similar hyperpolarising response to adenosine and other purinergic agents. In some cells the hyperpolarisation was preceded by a transient depolarising current. Further study (Lotan, Dascal, Oron *et al.*, 1985) revealed that the transient inward current reversed around -22mV and was therefore carried by Cl<sup>-</sup>. The hyperpolarising current reversed around -102mV and was carried by K<sup>+</sup>.

The K<sup>+</sup> current induced by adenosine was blocked by low doses of theophylline, and the potency sequence for the purinergic agonists was adenosine = AMP > ADP = ATP. These results suggest that the response is mediated by a P<sub>1</sub> purine receptor. The Cl<sup>-</sup> current induced by adenosine, however, was not blocked by theophylline and the agonist potency sequence was opposite to that of the K<sup>+</sup> current. Thus the Cl<sup>-</sup> response would seem to be dependent on a P<sub>2</sub> receptor.

### (iii) Other receptors

Further studies have revealed that several agonists produce a similar outward K<sup>+</sup> current these include corticotrophin-releasing factor (CRF), arginine vasopressin (AVP) (Moriarty, Gillo, Sealfon *et al.*, 1988) porcine vasoactive intestinal peptide (VIP) (Woodward & Miledi, 1987b), mammalian gonadotrophins (FSH and hCG) (Woodward & Miledi, 1987a), prostaglandins, oxytocin and human atrial natriuretic factor (Miledi & Woodward, 1989). All these responses appear to be mediated by distinct receptors which are coupled to a common intracellular messenger pathway.

There are several pieces of evidence which suggest that cAMP is the second messenger which produces these responses. Firstly, exposure to adenosine leads to a small rise in the intracellular cAMP level, which precedes the electrophysiological response (Lotan, Dascal, Oron *et al.*, 1985). Intracellular injection of cAMP was also shown to produce an outward current with a reversal potential of around -100mV. A similar outward current was seen after the application of the adenyl cyclase activator, forskolin. In addition a low concentration of forskolin potentiates the response to adenosine, possibly due to a forskolin induced enhancement of interaction between subunits of adenyl cyclase (Stinnakre & Van Renterghem, 1986). In addition, intracellular injection of protein kinase inhibitors suppresses the response to adenosine in a dose dependent manner (Lotan, Dascal, Oron *et al.* 1985). Thus the different receptors are all thought to stimulate adenylate cyclase causing an increase in



3',5'-cyclic monophosphate (cAMP) which in turn, probably through activation of cyclic AMP dependent protein kinase(s), regulates the gating of specific  $K^+$  channels.

The location of the receptors which induce this slow potassium current has been the subject of some debate. The response to these agents is removed after the oocyte has been defolliculated, either manually, or by treatment with collagenase. It was also shown that denuded oocytes lost the ability to respond to injection of cAMP or forskolin (Dascal, 1987). The question which remains is are the receptors in the follicular layer, and/ or the potassium channels, or are both on the plasma membrane and damaged by the process of defolliculation. As the  $K^+$  current responses are all abolished by defolliculation, it seems likely that the responses are located in the follicular cells. Thus when recording from within follicle enclosed oocytes cyclic nucleotide-activated  $K^+$  currents are only detected because follicular cells are electrically coupled to the oocyte by gap-junctions (Browne, Wiley & Dumont, 1978; Brown & Werner, 1984; Van den Hoef, Dictus, Hage & Bluemink, 1984).

## **(B) Oscillatory chloride currents.**

### **(i) The muscarinic response**

The first reported response to acetylcholine (ACh) in *Xenopus* oocytes consisted of a depolarisation which was either preceded or followed by a hyperpolarization, and was usually accompanied by large fluctuating depolarizations (Kusano, Miledi & Stinnakre, 1977). The response to ACh seemed to reside in the oocyte membrane because defolliculation by collagenase did not always abolish the response. Under voltage-clamp the response consisted of a depolarising inward current, with a rise in membrane conductance which was not abolished by removal of external  $Ca^{2+}$ ,  $Na^+$ , or  $Cl^-$ . The conclusion was that the inward current elicited by ACh was carried mostly by  $Cl^-$ . A later study (Dascal & Landau, 1982) which looked at the reversal potential confirmed this. Further study revealed that the muscarinic response had four components (Dascal & Landau, 1980;1982); A fast transient inward current (which may be absent during the winter months), a slow inward component (mostly due to chloride but with an underlying  $K^+$  current (Dascal, Landau & Lass, 1984)), large inward current fluctuations superimposed on the slow component and an occasional outward current which is sensitive to collagenase treatment.

Iontophoretic application of ACh evokes larger inward currents in the animal pole than in the vegetal (Kusano, Miledi & Stinnakre, 1982). This may be due to the non-uniform distribution of chloride channels or there may be a similar distribution of muscarinic receptors.

All the  $\text{Cl}^-$  response components following ACh application were suppressed by intracellular injection of the  $\text{Ca}^{2+}$ -chelator EGTA. On the basis of this Dascal, Gillo & Lass (1985) concluded that the ACh-evoked  $\text{Cl}^-$  currents were  $\text{Ca}^{2+}$ -dependent and as the application of calcium channel blockers externally had no effect on the response, that the  $\text{Ca}^{2+}$  had been mobilized from intracellular stores. This hypothesis was supported by the following findings;

1. Intracellular injection of  $\text{Ca}^{2+}$  also evoked a  $\text{Cl}^-$  response which consisted of a fast and a slow component, accompanied by  $\text{Cl}^-$  current fluctuations (Miledi & Parker, 1984; Dascal, Gillo & Lass, 1985)

2. Repetitive applications of ACh in a  $\text{Ca}^{2+}$ -depleted, high  $\text{Mg}^{2+}$  solution resulted in a progressive decrease in the amplitude of the response. This was interpreted as being due to a depletion of stores (Dascal, Gillo & Lass, 1985).

3. Intracellular injection of the  $\text{Ca}^{2+}$ -releasing messenger inositol trisphosphate ( $\text{IP}_3$ ) evoked a two component  $\text{Cl}^-$  current closely resembling the ACh response. Also application of ACh produced an increase turnover of polyphosphoinositides, and an increase in the intracellular concentration of  $\text{IP}_3$  (Oron, Dascal, Nadler & Lupu, 1985).

4. Injection of the non-hydrolysable analog of GTP, GTP $\gamma$ S, evoked a slow inward  $\text{Cl}^-$  current with superimposed current fluctuations (Dascal, Ifune, Hopkins *et al.* 1986). Such an injection could be expected to activate G-proteins, possibly the one coupled to phospholipase-C which leads to calcium mobilization.

Thus the ACh-evoked  $\text{Cl}^-$  current would appear to be mediated by the activation of G-proteins which leads to an increase in phosphoinositide turnover, the production of  $\text{IP}_3$  and the mobilization of  $\text{Ca}^{2+}$  from intracellular store(s).



(ii) The response to cholecystokinin (CCK)

The application of CCK to follicular oocytes produces an oscillatory inward current similar to that induced by ACh. The current was carried by  $\text{Cl}^-$  and was not abolished by defolliculation (Moriarty, Gillo, Sealton *et al.*, 1988).

(iii) The response to divalent cations.

Divalent cations such as  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  induce a similar oscillatory chloride current (Miledi, Parker & Woodward, 1988). These agents are believed to act on a common mechanism to the ACh response, which involves hydrolysis of inositol phospholipids, mobilization of intracellular calcium and activation of calcium sensitive chloride channels.

### SECTION 1.3

#### THE USE OF *XENOPUS LAEVIS* OOCYTES FOR THE EXPRESSION OF EXOGENOUS RECEPTORS AND ION CHANNELS

*Xenopus laevis* oocytes have been used for many years to study gene expression mechanisms (for review see Gurdon, 1974, Lane, 1981; Soreq, 1985; Dascal, 1987). In the course of these studies an important discovery was made. This was the demonstration that oocytes are capable of synthesizing exogenous proteins when injected with foreign messenger RNA (Gurdon, Lane, Woodward & Marbaix, 1971). Following the injection of mRNA exogenous membrane proteins are synthesized, processed and inserted into the membrane of the oocyte. The significance of this finding was not realised until the beginning of the 1980's, when the expression of functional neurotransmitter receptors in *Xenopus* oocytes was demonstrated (Sumikawa, Houghton, Emlage *et al.*, 1981; Barnard, Miledi & Sumikawa, 1982). This was soon followed by the expression of functional voltage gated  $\text{Na}^+$  and  $\text{K}^+$  ion channels (Gundersen, Miledi & Parker, 1983b).

This ability to synthesize exogenous proteins is not an exclusive property of *Xenopus* oocytes, as other cell types are capable of similar expression, for example, mammalian cell lines such as mouse COS cells (Dixon, Irving, Candelene *et al.*, 1987). These cells display a transient expression of exogenous receptors in a similar way to *Xenopus* oocytes and have been used recently to clone the substance P receptor (Hushey & Krause, 1990). Recently a further

system has been developed by Claudio, Green, Harman *et al.* (1987) in which mammalian fibroblasts are stably transduced with the desired receptor. These cells continuously express the receptor, allowing binding and single channel conductance studies. However, oocytes are the most popular system, perhaps because they are very efficient in expressing the foreign message (Soreq, 1985). Another important factor is the large size of the oocytes, up to 1.3mm in diameter. This makes the oocytes easy to isolate and allows penetration by several microelectrodes, for electrophysiological measurement and for injection of various substances.

### **The functional expression of receptors in *Xenopus* oocytes**

The list of receptors which have been functionally expressed in *Xenopus* oocytes is long, and steadily increasing. The receptors which have been expressed are shown in TABLE 1.

The receptors fit into two broad categories, those which activate second messengers (IP<sub>3</sub> and cAMP) and those which are ligand gated channels.

#### **(A) Receptors which activate second messengers**

These receptors are thought to have similar structures, consisting of seven helical trans-membrane regions (Lester, 1988). All are thought to have a cytoplasmic region which interacts with GTP-binding proteins (G-proteins) which have been shown to provide signal transduction for many cell surface receptors (Gilman (1987). It is believed that the expressed receptor interacts with a G-protein which is endogenous to the *Xenopus* oocyte since the injection of single cloned receptors (which could not introduce a G-protein from the source tissue) produce similar responses to receptors expressed following injection of mRNA. From then on the response is due to the activation of second messenger systems which occur in the native oocyte. The existence of endogenous G-proteins in the plasma membrane of eggs has been demonstrated by the use of cholera and pertussis toxins (Turner, Jaffe & Primakoff, 1987). Further evidence comes from the demonstration that microinjection of GTP $\gamma$ S causes a transient rise in the internal concentration of Ca<sup>2+</sup> which is very similar to that seen at fertilization (Swann, Ciapa & Whitaker, 1987).

The G-proteins are membrane associated heterotrimeric proteins composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. The  $\alpha$  subunit contains a guanine nucleotide binding site that in the non-activated state is occupied by GDP. The activation of a G-protein by a receptor results in the displacement of bound GDP for GTP. The GTP-

**Table 1.1** Receptors which have been expressed in *Xenopus* oocytes by the injection of mRNA

<u>AGONIST</u>	<u>ION</u>	<u>KINETICS</u>	<u>NOTES</u>	<u>REFS</u>
Acetylcholine	Cl-	long latency transient spikes oscillations	muscarinic pharmacology mimicked by IP3 injection blocked by intracellular EGTA desensitises cross desensitisation with 5-HT1c	Sumikawa et al 1984 Dascal et al 1986 Hirono et al 1987 Sugiyama et al 1985 Fukuda et al 1987 Kuba et al 1986
Angiotensin II	Cl-	long latency smooth currents and oscillations	increase in inositol phosphates	McIntosh & Catt 1987 Meyerhoff et al 1988
B1-adrenergic			causes a rise in cAMP level	Fricelle et al 1987
B2-adrenergic			causes a rise in cAMP level	Klobilka et al 1987
Bombesin	Cl-		releases internal Ca blocked by EGTA	Williams et al 1988 Moriarty et al 1988
Cholecystokinin	Cl-		releases internal Ca blocked by EGTA	Williams et al 1988 Moriarty et al 1988
GABA	Cl-	short latency, smooth	desensitises, GABAa pharmacology but not potentiated by benzodiazepines no effect of pertussis or cholera toxin	Smart et al 1987 Houamed et al 1984 Sigel et al 1988 Schofield et al 1987 Levitan et al 1988
Glutamate Quisqualate	Cl-	long latency transient spikes oscillations	no block with Joro spider toxin mimicked by IP3 injection cross-desensitisation with ACh attenuated by pertussis toxin, EGTA	Gundersen et al 1984 Sugiyama et al 1987 Lerma et al 1988 Fong et al 1988
Glycine	Cl-	short latency, smooth	blocked by strychnine, desensitises	Houamed et al 1984 Verdoorn et al 1989
Kainate	Na+ / K+	Short latency smooth	blocked by Joro spider toxin no effect of pertussis toxin, cholera toxin, or EGTA non-desensitising	Houamed et al 1984 Dascal et al 1986 Gundersen et al 1984 Lerma et al 1989 Fong et al 1988

<u>AGONIST</u>	<u>ION</u>	<u>KINETICS</u>	<u>NOTES</u>	<u>REFS</u>
L-glutamate	Cl-	long latency transient spikes oscillations	blocked by EGTA	Fong et al 1988
Neurokinin A	Cl-	long latency transient spikes oscillations		Harada et al 1987
Neurotensin	A) Cl- B)?	A) long latency transient spikes oscillations B) smooth	desensitises cross-desensitisation with ACh mimicked by IP3 injection blocked by intracellular EGTA	Hirono et al 1987 Parker et al 1986
NMDA	Na+ / K+	short latency smooth	non-desensitising potentiated by glycine voltage-dependent Mg2+ block	Verdoorn et al 1987/9 Lerma et al 1989 Fong et al 1988 Klackner et al 1988 White & Reisine 1990
Somatostatin			causes a reduction in a cAMP raised by B2 stimulation	
Substance K	Cl-	long latency transient spikes oscillations	desensitises	Harada et al 1987 Masu et al 1987
Substance P	A) Cl-	A) long latency transient spikes oscillations	desensitises	Parker et al 1986 Harada et al 1987
TRH	Cl-	long latency smooth currents and oscillations	increase in inositol phosphates	McIntosh & Catt 1987 Oron et al 1987 Meyerhoff et al 1988
Vasopressin	Cl-	long latency smooth currents and oscillations	releases internal Ca	Williams et al 1988 Meyerhoff et al 1988
5-HT	Cl-	long latency transient spikes oscillations	5-HT receptor pharmacology mimicked by IP3 injection blocked by intracellular EGTA desensitises cross-desensitisation with ACh	Dascal et al 1986 Lubbert et al 1987a,b Gundersen et al 1983a Takahashi et al 1987 Julius et al 1988

bound form of the G-protein then initiates a cellular response by altering the activity of specific enzymes. The number of receptors which can activate G-proteins is large, but the number of effector molecules is low. The type of G-protein activated then dictates the type of second messenger produced and the type of response seen in the oocyte.

### **Second messenger systems of the *Xenopus* oocyte.**

Second messenger systems of the *Xenopus* oocyte have been studied since the discovery of endogenous muscarinic,  $\beta$ -adrenergic (Kusano, Miledi & Stinnakre, 1977; 1982), and later purinergic responses (Lotan, Dascal, Cohen & Lass, 1982) in the oocyte. There appear to be at least two distinct second messenger systems, the  $IP_3$ - $Ca^{2+}$  and the cAMP systems, which are involved in the mechanism of response to these transmitters.

#### **(i) The cAMP system**

Several transmitters and hormones (such as norepinephrine through  $\beta$ -adrenergic receptors, adenosine through  $P_1$  receptors, and dopamine through  $D_1$  receptors) elevate the cAMP level by activating the enzyme adenylate cyclase, which converts ATP to cAMP (Beam & Greengard, 1976; Greengard, 1976; Kebabian & Calne, 1979; Onali, Eva, Orianas *et al* 1983; Fain & Malbon, 1979 & Satchell, 1984). In many biological system studied, cAMP produces its final effect through the activation of a cAMP-dependent protein kinase (A-kinase) (Greengard, 1976; 1978). A-kinase phosphorylates the target protein(s); in the few known cases of cAMP-dependent regulation of ion channels, the target protein is the channel itself or a closely associated protein (Ewald, Williams & Levitan 1985; Levitan, 1985). Such a response is initiated by activation of the G-protein,  $G_s$ , the stimulatory regulator of adenylate cyclase (Ross & Gilman, 1977).

Following the expression of  $\beta_1$  and  $\beta_2$  adrenergic receptors (Friele, Collins, Daniel *et al* 1987; Kolbilka, Macgregor, Daniel *et al* 1987), a rise in the cAMP level, measured by radio-immunoassay, has been demonstrated. In defolliculated oocytes, this rise in intracellular cAMP does not activate any ion channels. Hence the expression of receptors cannot be studied with

electrophysiology and must be studied by measuring the intracellular level of cAMP.

Several neurotransmitters and neuromodulators inhibit adenylate cyclase and suppress cAMP accumulation. Such an inhibition of adenylate cyclase is regulated by the inhibitory G-protein,  $G_i$  (see Johnson & Dhanasekaran, 1990 for review). Among these are ACh acting through muscarinic receptors, norepinephrine acting through  $\alpha_2$ -adrenergic receptors, dopamine at D2 receptors and opiate agonists (Robdell, 1980; Cooper, 1982).

Until recently, the expression of exogenous receptors which activate  $G_i$  could not be studied as such a study of the reduction in the level of cAMP within the cell first of all requires the cAMP level to be raised, which proved difficult. However, the cloning of the  $\beta_1$  adrenergic receptor (Dixon, Kolbilka, Strader *et al.*, 1986) produced a satisfactory means of raising the cAMP level. Using this it has been shown that expressed somatostatin receptors can decrease the level of cAMP in *Xenopus* oocytes (White & Reisine 1990).

#### (ii) The $IP_3$ - $Ca^{2+}$ system

Many hormones and transmitters regulate the intracellular level of  $Ca^{2+}$ , mostly causing an elevation. Such agents are ACh, serotonin, dopamine etc which act on endogenous receptors and have already been mentioned as adenylate cyclase inhibitors (Putney, 1978; Berridge & Irvine, 1984). This increase in  $[Ca^{2+}]_i$  may be a result of either  $Ca^{2+}$  entry through some receptor operated channels, or a mobilization of  $Ca^{2+}$  from intracellular stores. The molecular mechanism of the latter has been the subject of much study (Berridge & Irvine, 1984; Nishizuka, 1984) agonist binding involves the activation of a G-protein which then activates an enzyme called phospholipase-C (Gomperts, 1983; Cockcroft & Gomberts, 1985; Joseph 1985; Johnson, & Dhanasekaran, 1990). Until very recently the G-protein responsible for this activation had not been identified, but a recent paper by Moriarty, Padwell, Carty *et al.* (1990) has shown that  $G_o$  is the G-protein which is linked to phospholipase-C. Activated phospholipase-C catalyzes the breakdown of membranal phosphatidylinositol phosphatases, in particular phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ), to produce diacylglycerol (DAG) and inositol 1,4,5 trisphosphate ( $IP_3$ ) (Berridge & Irvine, 1984).



## Inositol trisphosphate (IP<sub>3</sub>)

The first study to demonstrate that IP<sub>3</sub> mobilised calcium came from Streb, Irvine, Berridge & Schulz (1983), who showed that the addition of IP<sub>3</sub> to permeabilised pancreatic acinar cells resulted in the release of Ca<sup>2+</sup> from these cells. In *Xenopus* oocytes, the injection of IP<sub>3</sub> has been shown to diffuse into the cytosol and releases calcium from intracellular stores (Busa & Nuccitelli, 1985). On the basis of fractionation experiments and the use of metabolic inhibitors IP<sub>3</sub> appears to release Ca<sup>2+</sup> from a membrane fraction which is a component of the endoplasmic reticulum (see Rana & Hokin, 1990 for review). This IP<sub>3</sub>-sensitive store would appear, from the experiments of Busa & Nuccitelli, (1985), where IP<sub>3</sub> was injected at different depths into the oocyte, to be close to the surface of the oocyte. Until recently the endoplasmic reticulum was considered to be the sole site of IP<sub>3</sub>-sensitive Ca<sup>2+</sup> storage within the cell. However, this has recently been questioned (Dunlop & Larkins, 1986; Guillemette, Balla, Baukel *et al.*, 1987; Payne & Fain, 1987). A study of IP<sub>3</sub> binding sites in liver has suggested that the IP<sub>3</sub>-responsive site may lie either in or in a fraction as yet inseparable from the plasma membrane. In addition a role for a discrete organelle called the calciosome which contains a calsequestrin-like protein has been suggested by histological work of Volpi, Krause, Hamashita *et al* (1988).

The elevated internal concentration of calcium then activates chloride channels which depolarise the cell. Thus the receptor activates the pathway which in the mature egg, upon insemination, causes the wave of depolarisation which prevents polyspermy (Jaffe, Cross & Picherell, 1986) by an as yet undetermined mechanism.

## Diacylglycerol

Diacylglycerol is the other product of PIP<sub>2</sub> breakdown (Berridge & Irvine, 1984). It diffuses in the membrane and activates the enzyme protein kinase-C (PKC). The activation of PKC would appear to have many properties (see review by Rana & Hokin, 1990), but one of the functions may be to inhibit calcium signalling and attenuate the phosphoinositide response. PKC may achieve this by phosphorylation of G-proteins (Katada, Gilman, Watanabe *et al.*, 1985). Such a process may be responsible for the desensitisation of receptors which is seen after activation.



The majority of receptors which have been functionally expressed in *Xenopus* oocytes activate this IP<sub>3</sub> pathway and elicit a Cl<sup>-</sup> current. For example, the 5-hydroxytryptamine (5-HT) receptor, and the ACh receptor (see Table 1) produce such chloride currents on stimulation with their ligands. This finding is not surprising since the list of agonists and tissues where formation of IP<sub>3</sub> has been documented is extensive (see Abdellatif, 1986; Sikar & Hokin, 1986 for review).

(B) Receptors which form ligand gated cation channels.

Some receptors also incorporate an ion channel and therefore activation of the receptor leads to direct opening of the channel. Such a response does not require the activation of endogenous second messenger systems and hence the latency of the response is shorter than that for a response which uses second messengers. Such receptor/channels can only be identified using electrophysiology and such studies have shown that the GABA receptor, for example, fits into this category (Houamed, Bilbe, Smart *et al.*, 1984).

### The functional expression of ion channels in *Xenopus* oocytes

There is similarly a large number of ion channels which have been expressed in the *Xenopus* oocyte membrane, these are listed in TABLE 2. The use of *Xenopus* oocytes has allowed the successful elucidation of the amino acid sequence of Na<sup>+</sup> (Noda, Suzuki, Takeshina *et al.*, 1984) and K<sup>+</sup> (Stuehmer, 1988) channels. Such studies have also made it possible to study the structure-function relations of the voltage-activated Na<sup>+</sup> ion channel (Stuehmer, Stocher, Sakmann *et al.*, 1989).

As mentioned at the beginning of this literature survey, the aim of this study was to express the GnRH and TRH receptors in the *Xenopus* oocyte and to measure this expression using electrophysiology. The eventual goal was to use the *Xenopus* expression system for the isolation of single clones from a size-selected cDNA library which encode the GnRH and TRH receptors. A number of studies have been carried out on gonadotropes and a cell line (GH3, which expresses TRH receptors) which suggested that GnRH and TRH receptors would be suitable for expression in the membrane of the *Xenopus* oocyte:

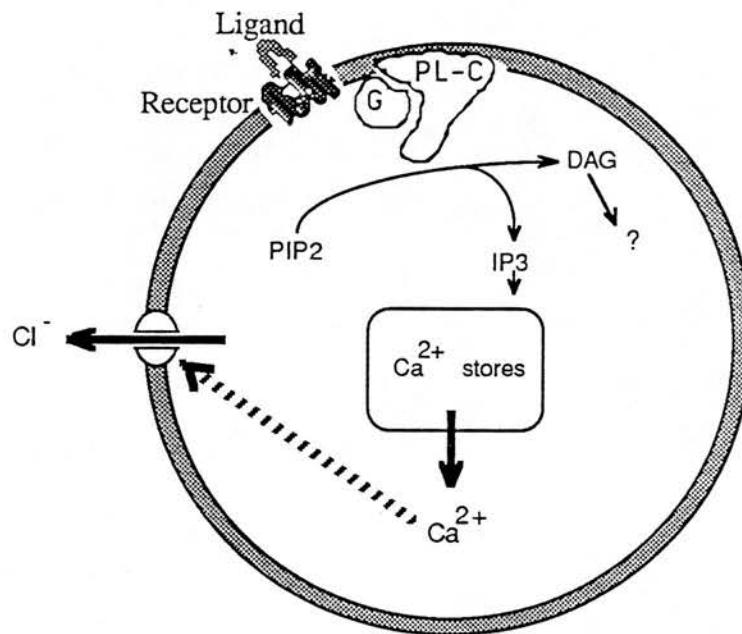
**Table 1.2** Ion channels which have been expressed in *Xenopus* oocytes by the injection of mRNA

<u>AGONIST</u>	<u>ION</u>	<u>KINETICS</u>	<u>NOTES</u>	<u>REFS</u>
Voltage-gated	Na+	Transient peak current ~ -10-0 mV	Kd~ 10nM for TTX Scorpion toxin removes inactivation Inactivation kinetics depend on fraction Three separate Na channels cloned, Na I, II and III	Gundersen et al 1983b Krafte et al 1988 Goldin et al 1986 Hirano et al 1985 Sigel et al 1987 Sigel et al 1987 Methfessel et al 1986 Noda et al 1986 Stuhmer et al 1988 Suzuki et al 1989
Voltage gated	Ca2+	Partial inactivation peak current at ~ +15mV	Recorded as a Ba 2+ current No effect of dihydropyridines Enhanced by phorbol esters	Lester et al 1988 Leanard et al 1987 Moorman et al 1987 Fournier et al 1989
Voltage-gated	K+	Transient Non-inactivating	'A' type current, insensitive to TEA Blocked by 4-Aminopyridine Delayed-rectifier type Blocked by external TEA May be more than one component	Gundersen et al 1983b Hoger et al 1987 Stuehmer et al 1987 Iversen et al 1988 Timpe et al 1988 Christie et al 1989
Voltage-gated	Cl-	Transient	Slowly inactivating, dependent on Ca Enhanced by phorbol esters	Gillo et al 1989

1. Activation of the TRH receptor has been shown to increase the breakdown of PIP<sub>2</sub> in GH3 pituitary cells ( Martin, 1983).
2. GnRH has been shown to stimulate the breakdown of PIP<sub>2</sub> in gonadotropes to produce DAG and IP<sub>3</sub> (Kiesel & Catt, 1984; Andrews & Conn, 1986).
3. GnRH has also been shown to increase the internal concentration of Ca<sup>2+</sup> in single gonadotropes using microspectrofluorimetry to monitor the Ca<sup>2+</sup> sensitive fluorescent chelator fura-2 (Shangold, Murphy & Millar, 1988).

Therefore it was thought likely that the GnRH and TRH receptors, if successfully expressed, would activate the G-protein (Go), which would stimulate the breakdown of PIP<sub>2</sub>, cause the production of IP<sub>3</sub>, which would release Ca<sup>2+</sup> from intracellular stores and cause the opening of Cl<sup>-</sup> channels, as summarised in Figure 1.1. Should such events occur GnRH and TRH receptors would therefore be suitable for a study of expression in the *Xenopus* oocyte using electrophysiological techniques.

**Figure 1.1** A diagrammatic representation of the events leading to Cl<sup>-</sup> channel opening, which occur upon the activation of a receptor which has been expressed in a *Xenopus* oocyte by the injection of mRNA.



- G - G-protein
- PLC - phospholipase C
- PIP2 - phosphoinositol biphosphate
- IP3 - inositol trisphosphate
- DAG - diacylglycerol

## GENERAL METHODS

### 2.1 SOLUTIONS

#### (A) Solutions for electrophysiology

The ionic composition of the standard solution for electrophysiology was (mM): NaCl, 115; KCl, 1; CaCl<sub>2</sub>, 1.8 and HEPES, 10, at pH 7.4. (Eidne, McNiven, Taylor et al. , 1988) The buffer HEPES has a pKa of 7.55 at 20°C and is reported to have no effect on the electrophysiological properties of membranes (Good, Winget, Winter, Connolly, Izawa and Singh, 1966).

For some experiments the composition of the bathing solution was altered. A list of the solutions used during the course of these experiments is given in Table 2.1. When the concentration of an ion was changed the osmolarity of the solution was maintained. Intermediate concentrations of any ion under study were obtained by mixing two parental solutions. Again, the concentration of all ions (except the ion under study and the ion which substituted for it) was kept constant.

Low Na<sup>+</sup> solution was obtained by either by equimolar substitution of NaCl with tetramethylammonium chloride (TMA-Cl) (Edwards, 1982) or by substitution by Tris<sup>+</sup> and the pH of the Na<sup>+</sup>-free solution was adjusted with HCl. The tonicity of the solution was made equal to that of the standard solution with Tris-Cl by considering dissociation of Tris (Hagiwara & Takahashi, 1974). Na<sup>+</sup>-free solution was mixed with standard solution to obtain desirable amount of Na<sup>+</sup>.

For calcium-free solutions the calcium-chelator Ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was used. In addition, in some solutions calcium was replaced with other alkaline earth cations, Ba<sup>2+</sup>, Sr<sup>2+</sup>, Mn<sup>2+</sup> and Mg<sup>2+</sup>.

Methansulphonate- was substituted for chloride in some experiments. This chemical is a good substitute because it is impermeant, fully dissociated at pH 7.4, does not require a change in the external concentration of calcium and does not change the pH (Sharpe and Thomas, 1981).

In most cases the experiments were performed in standard solution and all data (unless otherwise stated) refer to this solution.

**Table 2.1** Solutions used for electrophysiological recording from *Xenopus* oocytes.



Solution	NaCl	KCl	CaCl2	MgCl2	NaOH	KOH	Methan- Sulphonate	EGTA	TMA+	BaCl2	MnCl2	SrCl2	HEPES
Standard (Mg-free)	115	2	1.8										10
Standard	115	2	1.8	1									10
Low Cl-			1.8	1	115	2	117						10
High K+	115	10	1.8	1									10
Low Na+	38.3	2	1.8	1				76.7					10
Ca-free	115	2						2					10
Ca-free +Mg	115	2		1.8				2					10
Ca-free +Ba	115	2						2		1.8			10
Ca-free +Mn	115	2						2			1.8		10
Ca-free +Sr	115	2						2				1.8	10

Units mM

Other chemicals added to these basic solutions were:

8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), N-phenylanthranilic acid (NPAA) dissolved in ethanol (final concentration of ethanol 0.1% V/V), ionomycin, antimycin A, oligomycin, 1,2-bis-12-aminophenyloxylethane-N,N,N',N'-tetra acetic acid BAPTA/AM and forskolin from Calbiochem San Diego USA; 9-anthracine carboxylic acid (9-Ac) were dissolved in dimethyl sulfoxide (DMSO) (maximum final concentration of DMSO was 0.2% (v/v), oxalate, methansulphonate-, cadmium chloride, caesium chloride and hydroxide from Aldrich Chemicals;

caffeine , chlordiazepoxide ionomycin, 2,4 dinitrophenol (DNP) tetraethylammonium chloride (TEA-Cl), oligomycin, pertussis toxin (PTX), 1-octanol, Tris<sup>+</sup>, ruthenium red, sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), atropine, theophylline,adenosine triphosphate (ATP), from Sigma, St. Louis, USA;

Ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), tetramethyl ammonium chloride (TMA-Cl), tetramethyl ammonium hydroxide (TMA-OH) and 4,4'-isothiocyanatostilbene-2,2' disulphonic acid (DIDS) dissolved in DMSO, final concentration of DMSO 0.1% (v/v) from Fluka.

## (2) Agonist solutions

The following agonists, dissolved in the normal solution were used as receptor ligands:

Gonadotrophin-releasing hormone (GnRH) and the GnRH agonist, buserelin ((D-Ser(But)6,Pro9-N-ethylamide)GnRH) were obtained from Hoechst AG, Frankfurt, FRG;

the GnRH antagonist (N-Ac-D-2-naphthylalanine1,D-pCl-Phe2,D-Trp3,D diethyl hArg6 ,D-Ala10)GnRH, was a gift from Syntex Research, Palo Alto, CA, USA;

Thyrotrophin-releasing hormone (TRH) was obtained from Cambridge Research Biochemicals, Cambridge, UK;

Acetylcholine chloride (ACh), N-acetyl-5-methoxy-tryptamine (Melatonin),Bovine (amino acid fraction 1-29 and 1-44), human (1-44), porcine (1-44) and rat (1-29 and 1-44) Growth hormone releasing factor (GRH) and 5-hydroxytryptamine (5-HT) were purchased from Sigma.

Dopamine was purchased from Fluka.

These agonist solutions were added to the oocyte by perfusion. The lag-time of the perfusion apparatus was approximately 2 seconds.

## 2.2 PREPARATION OF POLY (A)+ messenger RNA.

The preparation of poly adenylate mRNA is summarised in figure 2.1. The anterior pituitary gland was removed from <sup>approximately 100</sup> adult female rats of the Sprague-Dawley strain and a preparation of RNA was extracted following the method of Chomczynski and Sacchi (1987). This RNA preparation contains messenger RNA (mRNA), ribosomal and transfer RNA and is referred to as total RNA.

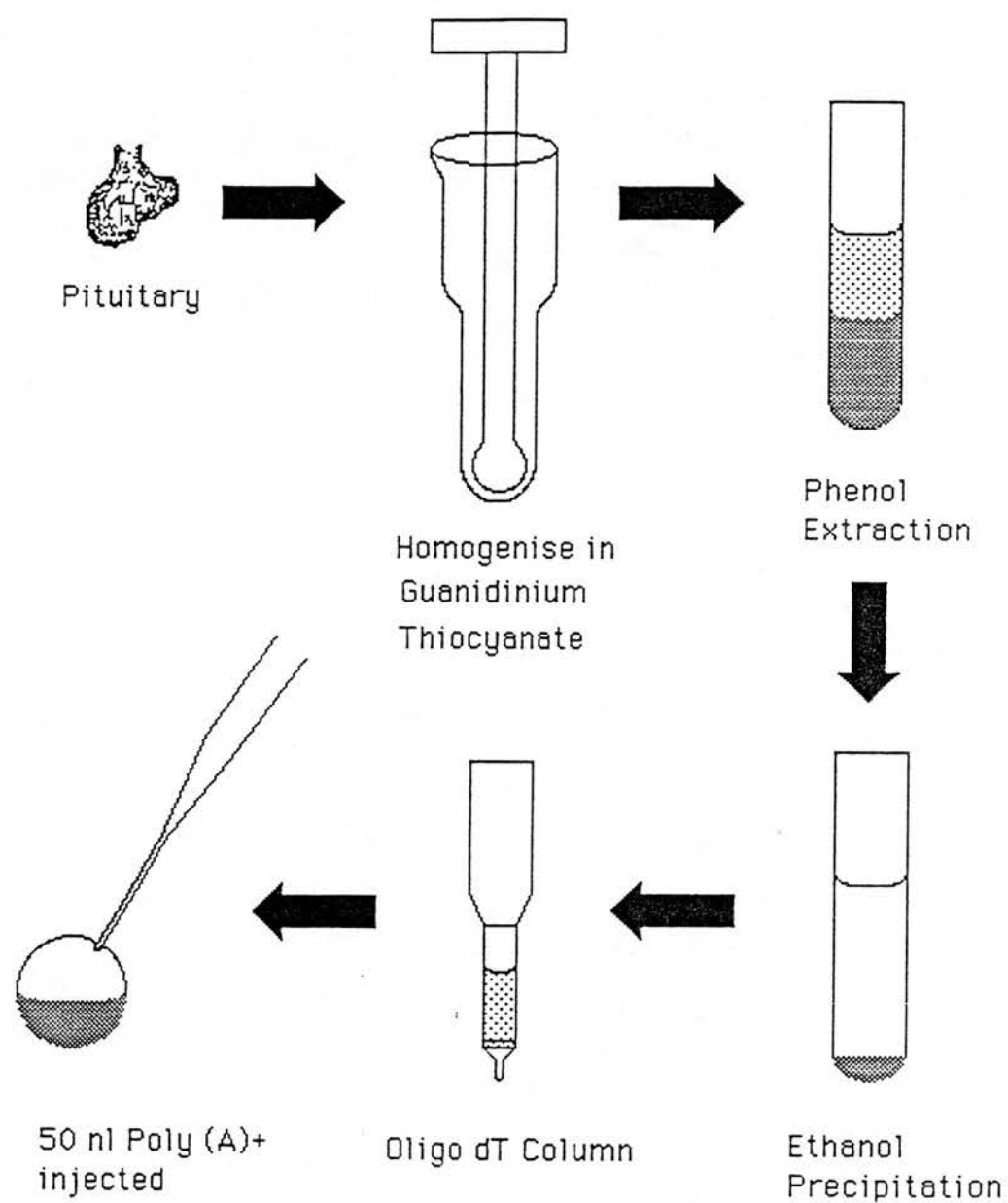
A poly (A)+-enriched mRNA fraction of the total RNA was obtained by oligo(dT) affinity chromatography (Maniatis, Fritsch and Sambrook, 1985). Messenger RNA with a poly adenylated tail is more likely to be expressed in the oocyte, as the poly (A)+ tails conveys some protection to the mRNA. This fraction of mRNA was then dissolved in sterile deionised water at a concentration of 1mg/ml and stored at -70°C until required. Eight batches of poly (A)+ mRNA preparations were used for experiments.

## 2.3 COLLECTION OF OOCYTES

Adult female *Xenopus-laevis* were obtained from Snake Farm, Fish Hoek, South Africa and maintained in a constant light-dark cycle (16hr light: 8hr dark) at 19°C. A donor was anaesthetised with either 3-aminobenzoic acid ethylester (tricaine; 0.1%) (Brown, 1970) or by hypothermia, using ice (Dumont 1972). A small incision was made on one side of the abdomen and part of the ovary containing oocytes teased out. The wound was then sutured and the post-operative frog returned to a water bath. Frogs could be repeatedly operated on but a period of no shorter than six weeks was allowed between operations.

After such a procedure the oocytes are massed together and surrounded by a number of cellular and non-cellular layers (Dumont and Brummet, 1978). Such oocytes are termed follicular oocytes and were used to study receptor expression with conventional intracellular recording and also for two-electrode voltage-clamp when receptors endogenous to the follicular layer were being studied. The follicular cell layer is electrically connected to the plasma membrane via gap junctions (Browne, Wiley & Dumont, 1978; Werner, Miller, Azarnia & Dahl, 1985)). Before recording could take place oocytes were singled out from the piece of ovary by cutting the connection of the epithelial layer to the ovary wall.

For two electrode voltage clamp studies of expressed receptors the outer follicular layers were removed either mechanically with forceps (Masui, 1967)



**Figure 2.1** Preparation of poly (A)+ mRNA

or by treatment with collagenase (2mg/ml) (Sigma) in a calcium-free bathing solution (which contained 1mM Mg<sup>2+</sup> to prevent deterioration of the oocytes), and gently agitated at room temperature for 1-1.5hrs, to aid defolliculation. The composition of this calcium free solution ("defolliculating" solution) is taken from Botton et al (1989) and is shown in Table 2.2. Defolliculation was assured by observation under a microscope (X3)

Defolliculated or follicular oocytes were then washed and maintained overnight at 19°C in modified Barths solution, shown in Table 2 (Aoshima, Iio, Anan et al. 1987) containing penicillin (100µg/ml)(Gibco), streptomycin (100ug/ml)(Gibco), sodium pyruvate (0.25mM) and ficoll (2%)(Sigma) before microinjection of mRNA.

## **2.4 MICROINJECTION OF mRNA INTO XENOPUS OOCYTES.**

The poly (A)+ mRNA was thawed at 70°C for three minutes and each oocyte was injected with 50nl (50ng) of this solution using a pressure injection system (Microlab M, Hamilton. Switzerland). Each oocyte was injected at the equatorial band or into the vegetal hemisphere. The oocytes were then incubated in modified Barth's solution for at least 48hr at 19°C before electrophysiological recording. Control oocytes were either untreated or injected with 50nl of sterile deionised water.

## **2.5 ELECTROPHYSIOLOGICAL RECORDINGS**

The expression of GnRH and TRH receptors took at least two days. Therefore oocytes were tested 2-6 days after the injection of mRNA. Two methods of electrophysiological recordings were used in this study and they will be described separately:

- (i) Intracellular recording.
- (ii) Two electrode voltage clamp.

### **(i) Intracellular recording**

This was performed on follicular oocytes i.e. not defolliculated. Membrane potential recording electrodes were pulled from fibre containing capillary tubes

**Table 2.2** Solutions for the incubation and defolliculation of *Xenopus* oocytes

<u>Constituents</u>	Modified Barth's	Ca-free, defolliculation solution
NaCl	88	96
KCl	1	2
CaCl <sub>2</sub>	0.41	—
MgCl <sub>2</sub>	—	1
NaHCO <sub>3</sub>	2.4	—
MgSO <sub>4</sub>	0.82	—
Ca(NO <sub>3</sub> ) <sub>2</sub>	0.33	—
HEPES	10	10

Units: mM

(Clark Electromedical Instruments; GC200F-15) to a DC resistance of 20-50M $\Omega$  (when filled with 3M KCl), using an electrode puller (Narishige, Japan.Type PE2). This microelectrode was connected to the input of a high impedance preamplifier (Dagan 8100).

An oocyte was then placed in a chamber with a fine, inert nylon mesh fixed in the bottom with formalin (to prevent the oocyte from moving) and bathed in standard solution. This chamber was on the headstage of a microscope (Olympus SZ, Japan) illuminated by a fibre optic lamp (KL1500, Schott) to avoid AC noise, on a locally made vibration free table, inside a Faraday cage. A glass microelectrode was inserted into the oocyte using an hydraulic micromanipulator (Narishige, Japan.Type MO5) and penetration was sometimes aided by over compensation of the negative capacitance of the preamplifier. This provided measurement of the membrane potential.

Only oocytes (either control or mRNA injected) showing resting membrane potentials more negative than -50mV were used. Occasionally a second intracellular microelectrode (20-40M $\Omega$ ) was inserted to administer current pulses from a Devices isolated stimulator (2533; Devices Ltd.) driven by a digitimer (D4030; Devices Ltd )for the measurement of the membrane resistance.

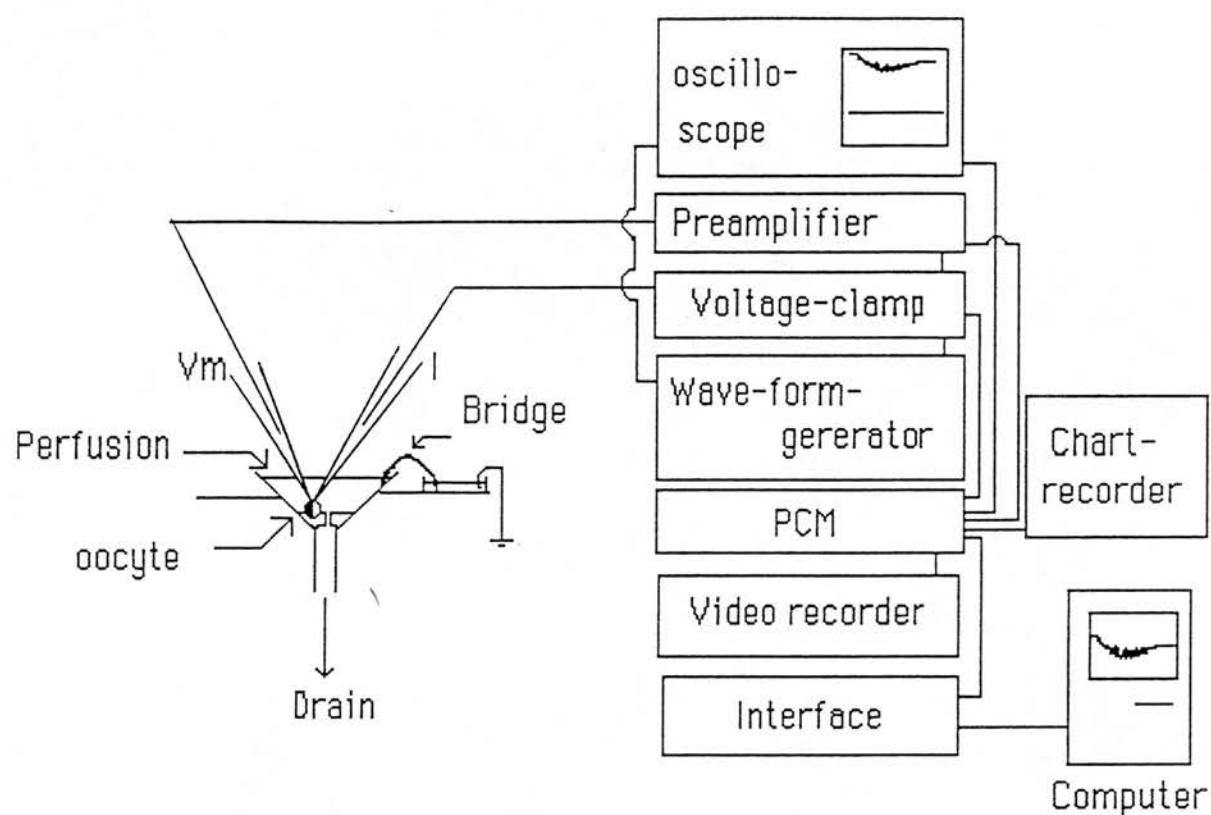
#### (ii) Two-electrode voltage clamp

This was performed on both follicular and defolliculated oocytes. Membrane potential recording electrodes were pulled from fibre containing capillary tubes (Clark Electromedical Instruments; GC200F-15) to a resistance of 3-10 M $\Omega$ , when filled with 3M KCl. This microelectrode was connected to the input of a high impedance preamplifier (circuit design in section2.3) and from this to the voltage input of a voltage-clamp machine (circuit diagram in section2.3).

Electrophysiological experiments were performed on single oocytes (follicular or denuded). An oocyte was placed in a chamber containing normal solution, constructed from a 50ml syringe (Plastipak, Becton-Dickinson, USA) containing an inert plastic which had a small well in it for the oocyte to rest in and an out-let tube for drainage (see Fig 2.2). This chamber was on the headstage of a microscope

(Olympus, SZ, Japan) illuminated by a fibre optic lamp (KL1500-T Schott) on a vibration free table (Ealing electro Optics;Holliston, MA. USA; U-frame vibration isolation system), inside a Faraday cage.





**Figure 2.2** Electrophysiological set-up for voltage-clamp

The membrane potential recording microelectrode was inserted into the oocyte using an hydraulic micromanipulator (Narishige, Japan. Type MO5) and by overcompensation of the negative capacitance. A second intracellular "current" microelectrode ( $0.7\text{--}1.5\text{M}\Omega$ ) was then inserted to inject current from the current output of the voltage-clamp machine (see Fig.3 for circuit diagram) to maintain the oocyte at the desired membrane potential (holding potential). Oocytes were routinely clamped at  $-60\text{mV}$  by the use of a conventional voltage clamp technique. A value of  $-60\text{ mV}$  was chosen because this was around the mean value of the resting membrane potential. The bath was connected to the ground Ag/AgCl electrode through a bridge.

Only oocytes (either control or mRNA injected) showing membrane potentials more negative than  $-20\text{mV}$  after insertion of the second microelectrode and which had a holding current of less than  $100\text{ nA}$  when voltage-clamped at  $-60\text{mV}$  were used. At the end of each experiment the potential drift of the voltage electrode was checked. As a rule, the drift was less than  $+3\text{mV}$ , otherwise the results were discarded.

For both the intracellular recording and the two-electrode voltage clamp the membrane potential and the membrane current were digitised by a Digital Audio Processor (Sony, PCM-701 ES, Japan sampling frequency  $44.1\text{ kHz}$ ) modified according to Lamb (1985) to accept DC signals and stored on video tapes using a conventional video recorder (Panasonic, NV-G25). There are several advantages in using a digital pulse code modulator (PCM) and video system in comparison with an analogue FM tape system. The video system has a wider dynamic range and a greater frequency response. The signal to noise ratio is also much higher for a video system. One problem with a PCM is that during its modification to accept a DC signal a problem with DC shift was introduced (this is temperature dependent and can be overcome by allowing the PCM time to warm up. The PCM was warmed up for three hours until its DC level became steady.

The data was then filtered with a 4-pole Butterworth filter (Kemo; VBF4) at  $30\text{Hz}$  and displayed simultaneously on a dual beam storage oscilloscope (Tetronix 5113, with a differential amplifier 5A22N, dual trace amplifier 5A18N and dual trace time base 5B12N) and also on a chart recorder (Graphtec, Tokyo, Japan; 6335). Analysis of data was performed by both measurement of traces from the chart recorder by hand and by feeding the stored signal, digitised at a sampling frequency of  $2\text{--}5\text{ Hz}$  to a computer (Macintosh Plus, USA) via an

interface (Analogue Highway, 3D Digital Design and Development Limited, London).

#### Measurement of the reversal potential.

The reversal potential of a response (the membrane potential at which no current flows through the membrane) was obtained in two ways;

1. The holding potential was changed in a step-wise manner, each potential being maintained for approximately two minutes. The direction of the current was monitored at each membrane potential (Kusano, Miledi & Stinnakre, 1982)
2. The ramp method as described by (Dascal, Landau & Lass, 1984) was applied. A ramp-like change in the membrane potential superimposed on the "steady" holding potential was obtained by feeding a ramp command from the output of a function generator (Tetronix; FG 501) to the command input of the voltage-clamp machine. The slope of the ramp was between 100mV/s and 150mV/s. The resulting changes in the membrane current and potential were stored on the oscilloscope. The reversal potential of a response to an agonist was measured by, firstly applying a ramp before the application of an agonist and secondly, during the response. The reversal potential was determined by the cross-over point of the two curves i.e. the membrane potential at which the application of agonist does not cause a net change in the membrane current.

#### Measurement of membrane resistance and capacitance.

The membrane resistance was measured under voltage-clamp conditions by changing the membrane potential in a linear region of the current/voltage relationship (see section 1.1) and measuring the change in the holding current, the membrane resistance was then calculated using Ohm's law.

The membrane capacitance could be measured in two ways, both of which were measured under current-clamp conditions. This required the use of the current-clamp circuit of the voltage-clamp machine (see section 2.6)

(i) Current pulses of different amplitude were applied to an oocyte, the resultant changes in the membrane potential were plotted against the current amplitude. From this the time constant ( $\tau$ ) of the membrane could be measured. Using this value and the membrane resistance ( $R$ ) the capacitance of the membrane ( $C$ ) could be calculated ( $\tau=RC$ ).

(ii) When a relatively brief current pulse ( 10 msec) compared with the time constant of the oocyte membrane (about 300 msec) was applied, the membrane potential showed an almost linear change, which represented  $dV/dt$ . By dividing the value of applied current (I) by  $dV/dt$ , the membrane capacitance was obtained ( $C=I \, dV/dt$ ) (Yamashita, 1982).

## 2.6 CONSTRUCTION OF ELECTROPHYSIOLOGICAL MACHINES.

For the voltage-clamp experiments two machines were constructed; a preamplifier and a two-electrode voltage-clamp system.

### (a) A preamplifier

My circuit design for a preamplifier is shown in Fig 2.3.

The probe (A) consists of a unitary gain voltage follower made from a high input resistance operational amplifier (Analogue Devices, AD503,  $R_{in}: 10^{11} \, \Omega$ ). This was chosen because it has a very small leakage current (15pA Max). The output of A conveys the membrane potential ( $V_m$ ) of the oocyte and the offset voltage ( $\Delta V$ ) due to the liquid junction potential, tip potential etc, which should be cancelled.

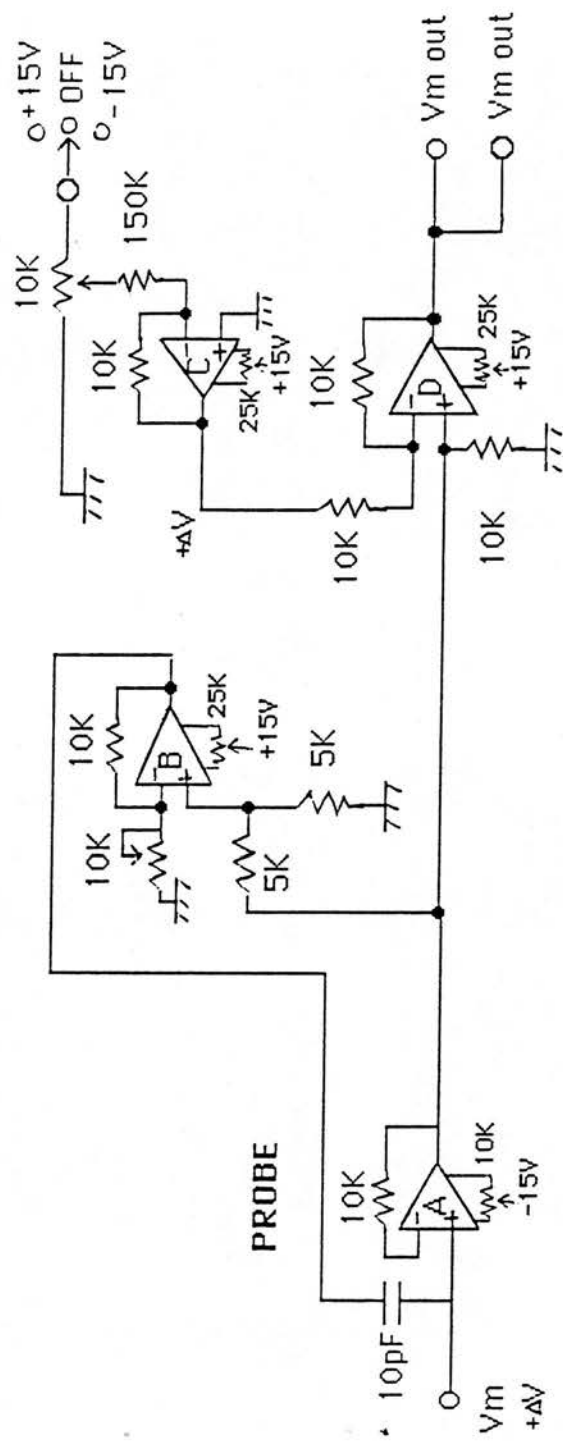
The offset voltage is cancelled with (C), which can adjust the DC level by  $\pm 1V$ . Any stray capacitance was cancelled with (B) which in conjunction with the 10pF capacitor in the probe, forms a capacitance compensation circuit. Appropriate compensation of the capacitance will aid voltage-clamping. In addition to this, over-compensation of the capacitance produces an oscillation of the microelectrode tip which aids penetration of the oocyte.

The operational amplifier (D) (a differential amplifier) receives  $V_m$  and  $\Delta V$  from the microelectrode to its non-inverting input . (C) creates the offset voltage ( $\Delta V$ ) and feeds it to the inverting input of (D). (D) cancels the difference between its two inputs and hence the output from (D) provides the membrane potential of the oocyte. All the operational amplifiers (except A) are type LF356 (National Semiconductors, input resistance 10 M $\Omega$ , maximum output  $\pm 13V$ ).

### (b) A two-electrode voltage-clamp system

The principle of voltage clamp is that there is a central operational amplifier, whose inputs are the membrane potential and the command voltage. The operational amplifier makes the difference between these two zero. Thus, the

**Figure 2.3** Circuit diagram of the pre amplifier



A - AD503

B-D - LF356

All resistor values are  $\Omega$

membrane potential follows the command voltage by injecting current into the cell.

The circuit diagram of such a system, designed by Drs K. Takahashi, M. Yoshii and S. Yoshida is shown in Figure 2.4).

The function of each operational amplifiers in this circuit diagram is :

F is a high impedance voltage follower (as for the preamplifier). This electrode can also measure the membrane potential ( $V_{out}$ ) to check whether the electrode has penetrated the cell and check the condition of the space-clamp.

E is the capacitance compensation circuit, which cancels stray capacitance and allows oscillation of the microelectrode (as for the preamplifier).

A is a summing amplifier to provide the command input and to change the holding potential.

C is the central operational amplifier which receives  $V_m$  and  $-V_c$  to its inverting input. The non-inverting input is at the ground level and the operational amplifier makes the inverting input at the ground level by injecting current into the cell. This operational amplifier also controls the gain, so that the command voltage amplitude can be accurately followed. It also controls the capacitance by minimising the capacitive current of the membrane. Unless restricted to within a short period (of less than 500  $\mu\text{sec}$  in our case), the capacitive current would mask the ionic current. Thus, a study of the ionic current is possible.

D is a differential amplifier which measures the voltage drop across the 1MO resistor which is connected to current-injecting electrode. Thus a voltage drop of 1V is due to the passage of  $1\mu\text{A}$ . As the maximum output of this amplifier is 13V the maximum current which can be injected is  $13\mu\text{A}$ .

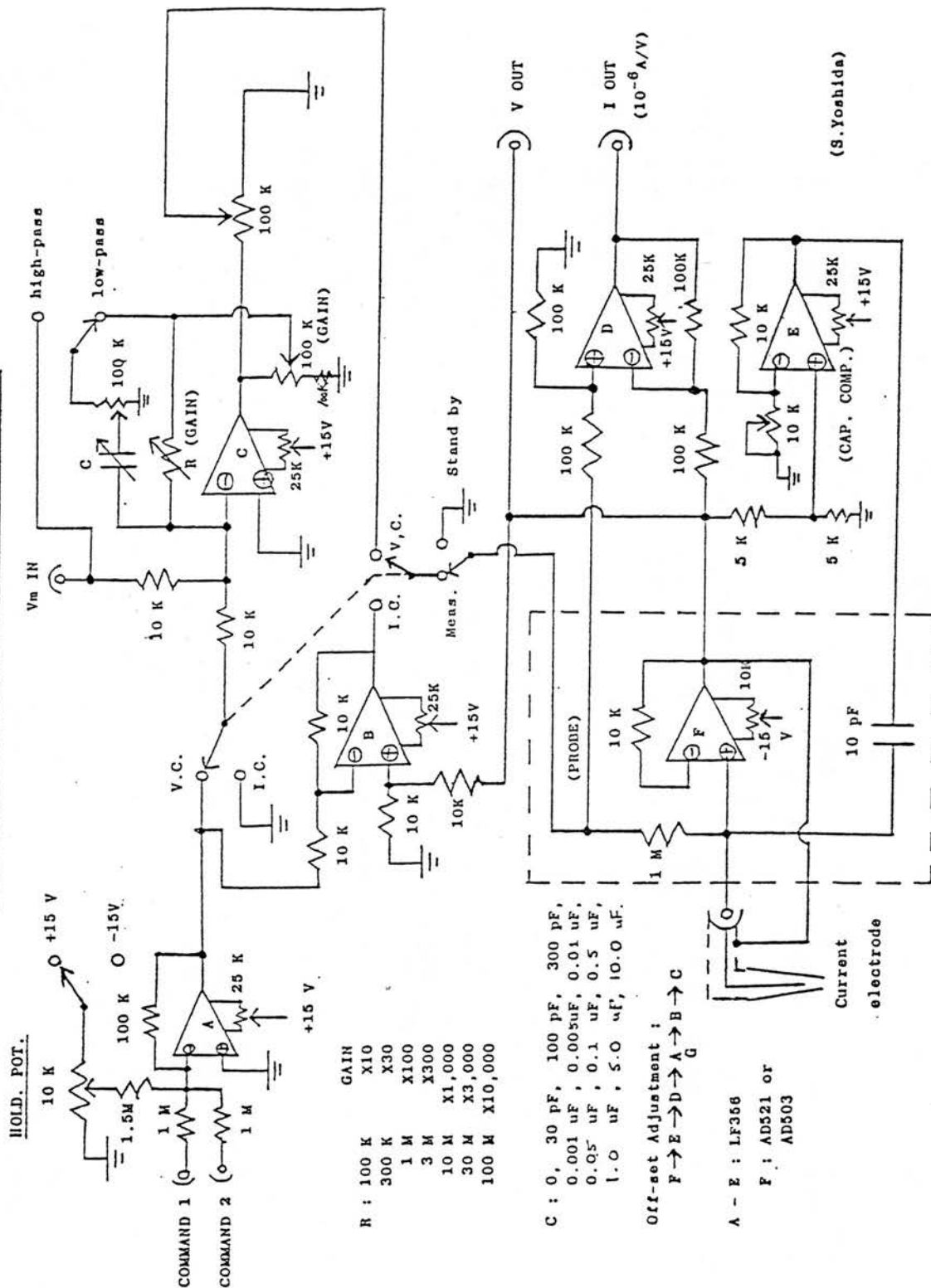
B is not involved in voltage clamping.

This circuit produces a voltage clamp system which has a settling time of less than 500  $\mu\text{sec}$  and can detect current of nA order.

**Figure 2.4** Circuit diagram of the two-electrode voltage-clamp and current clamp system.



# TWO-MICROELECTRODE VOLTAGE-CLAMP SYSTEM (March, 1987)



### (c) Current-clamp

As shown in the circuit diagram (Figure 2.3) the machine also includes a current clamp system which uses the differential operational amplifier B instead of C as the central amplifier. In this case the inverting input of B receives the inverted command pulse (-P) and the non inverting input receives  $V_m$  from the output of F. The output of B is then  $V_m - P$ . The voltage drop across the  $1M\Omega$  resistor which is connected to the current injecting electrode is therefore P. This voltage drop across the  $1M\Omega$  resistor is kept constant regardless of the membrane potential and therefore a constant current is injected into the cell. Thus the cell is current clamped. This circuit was used to measure the membrane capacitance.

### (e) A one point grounding system

In Britain the grounding system of a machine is provided by the earth pin of the plug which is connected to earth via the wall socket. As so many machines are used for electrophysiology the probability of ground loops being set up is large. As we are looking at very small changes in potential or current the noise introduced by such ground loops should be removed.

For this reason a one point ground system was used in the laboratory. This involves the use of a central ground board. Firstly all the machines were disconnected from the ground by removing the connection to the earth pin of the plug. The chassis ground of each machine is then connected to the rack, on which they are mounted. The rack is in turn connected to a low resistance central ground board, which consists of a thick copper wire with many connectors. This central board is at ground because it is connected to the mains ground via a thick wire.

In addition to the chassis ground all electrophysiological machines have a signal ground which gives a reference potential to operational amplifiers. These should all be connected and then a single connection made to the central ground board. The central ground board also grounds the Faraday cage and the microscope. Following this procedure produces a marked reduction in the noise level.

### SECTION 3

#### THE EXPRESSION OF ANTERIOR PITUITARY RECEPTORS, STUDIED USING CONVENTIONAL INTRACELLULAR RECORDING.

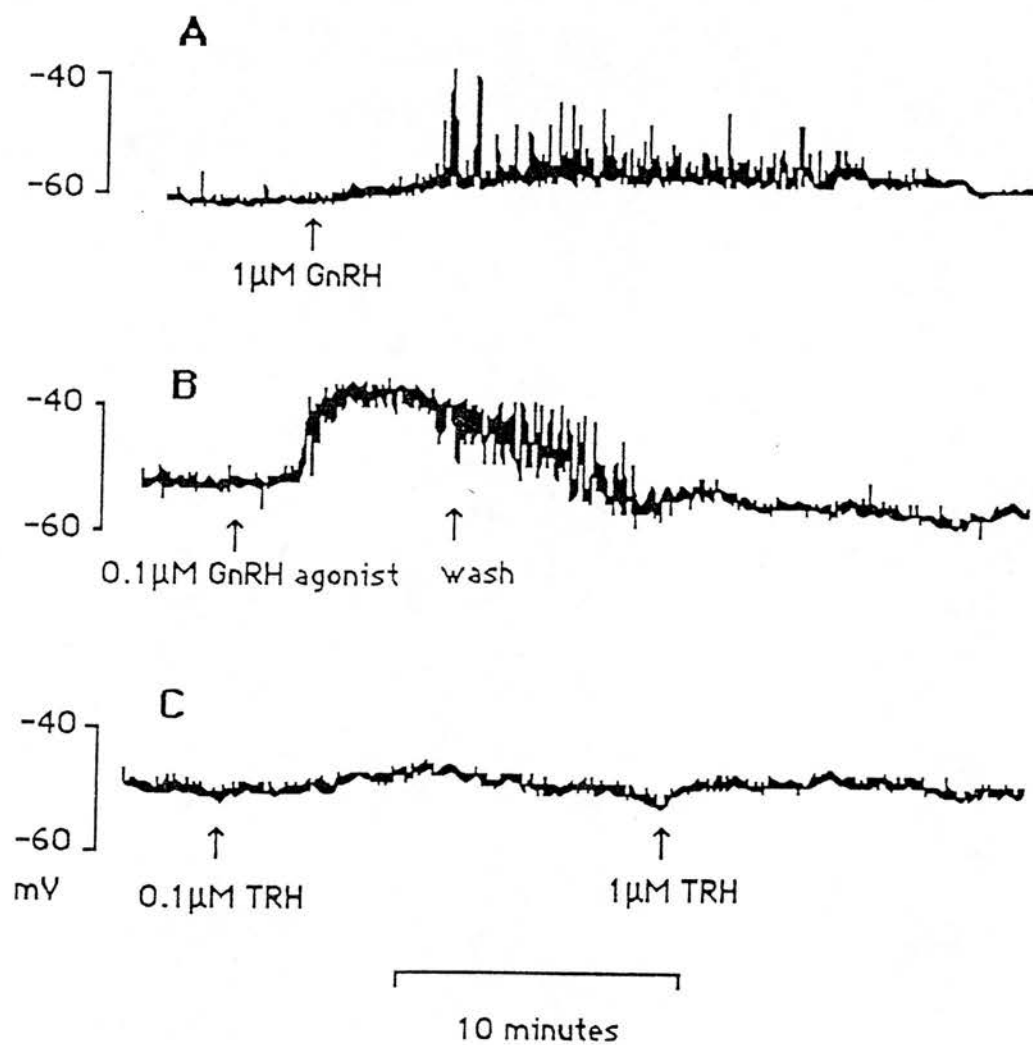
This section reports an initial study of the expression of gonadotrophin releasing hormone (GnRH) and thyrotrophin releasing hormone (TRH)<sup>receptors</sup> in *Xenopus* oocytes as carried out using conventional intracellular recording techniques. Such a strategy has been utilized for initial studies of expression of a number of receptors, including the acetylcholine receptor (Barnard, Miledi & Sumikawa, 1982) and also in the isolation of a single clone encoding the substance K and serotonin (5-HT<sub>1c</sub>) receptors (Masu, Nakayama, Tamaki *et al.*, 1987; Julius, MacDermott, Axel *et al.*, 1987).

The results from the study of 70 oocytes presented in this section, reveal that the GnRH and TRH receptors can be functionally expressed in the membrane of the *Xenopus* oocyte following injection of poly (A)+ mRNA from the rat anterior pituitary gland. The application of TRH and GnRH caused a depolarising response in the membrane. Included in this section are studies of the effect of a GnRH agonist (buserelin) which is used clinically in the treatment of breast and prostatic cancers (Frazer & Baird, 1987), GnRH and TRH receptor antagonists and organic channel blockers.

##### (A) The response to activation of the GnRH receptor.

The addition of GnRH caused marked membrane electrical responses in 65% of the injected oocytes. An example of such a response is shown in Figure 3.1A. Numerous episodes of depolarisation, of between 5-15mV in amplitude were observed with a latency of 4 min after the continuous application of GnRH (1 $\mu$ M) to the bathing solution. These depolarisations slowly declined in amplitude and frequency over the next 10-15 min, despite continuous exposure to GnRH. The same oocyte, after several minutes of washing, was then exposed to a super-agonist of GnRH (buserelin), at the lower concentration of 0.1 $\mu$ M. This caused the dramatically enhanced response which is shown in Figure 3.1B. The response to buserelin consisted of a sustained depolarisation to -30mV, which had a shorter latency. This response was reduced by washing the oocyte, when the response consisted of individual depolarisations (similar to those induced by GnRH). In contrast, no response was observed in this

**Figure 3.1** Typical changes in the membrane potential of a single *Xenopus* oocyte injected with 50ng of poly (A)+ mRNA prepared from rat pituitary gland. (A) 1  $\mu$ M GnRH, (B) 0.1 $\mu$ M GnRH agonist (Buserelin) and (C) 0.1 and 1 $\mu$ M TRH were added to the medium bathing the oocyte. The arrows indicate the onset of ligand administration, or its removal. In this and all other Figures in Section 3, the mV scale refers to the membrane potential of the oocyte



oocyte when exposed to 0.1 or 1 $\mu$ M TRH (Figure 1C). However, further responses to GnRH or busserelin could be elicited and hence the oocyte was still responsive. Thus the expression of GnRH is not linked to the expression of TRH.

The minimum concentration of GnRH which could elicit a response was 1nM. The depolarising membrane fluctuations became larger in size and appeared more frequently with increasing ligand concentration. At the maximal concentration of GnRH agonist tried (1 $\mu$ M) the response consisted of an initial sustained depolarisation, which resulted from the summation of individual depolarisations, decayed with time into individual depolarisations.

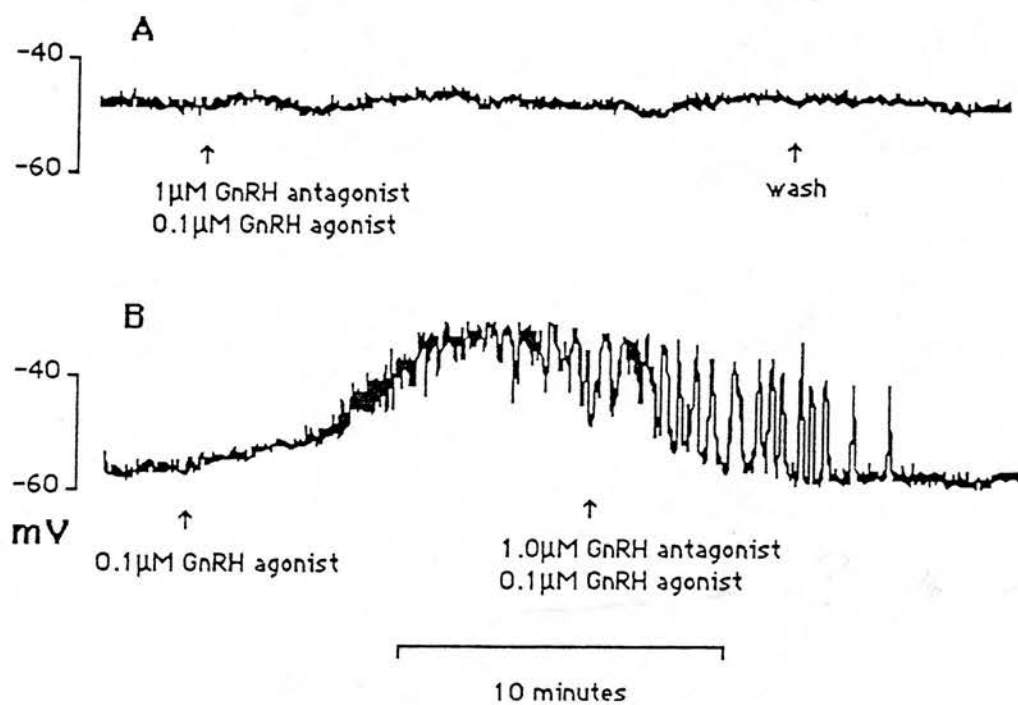
The membrane depolarisations induced by GnRH or its agonist were eliminated by the application of a GnRH antagonist ((N-Ac-D-2-naphthylalanine1,D-pCl-Phe2,D-Trp3,D diethyl hArg6 ,D-Ala10)GnRH). An example of such antagonism is shown in Figure 3.2. In this oocyte, the application of 0.1 $\mu$ M GnRH in the presence of the antagonist (1.0 $\mu$ M) produces no depolarisations (Figure 3.2A). In the same oocyte, following extensive washing for several minutes, application of the GnRH agonist elicits the typical continuous depolarisation, which is followed by individual depolarisations, until the application of the antagonist produces an inhibition of the response, despite the continued presence of the GnRH agonist (Figure 3.2B) No effect was seen upon the application of the antagonist alone.

The response elicited by GnRH also showed some desensitization. Upon continuous application of GnRH the response gradually disappeared over approximately 20 minutes. In addition, after the first application of GnRH, even if the oocyte was extensively washed, a later challenge with GnRH would elicit a smaller response with a longer latency or would be unable to respond to further application of GnRH.

#### **(B) The response to the activation of the TRH receptor.**

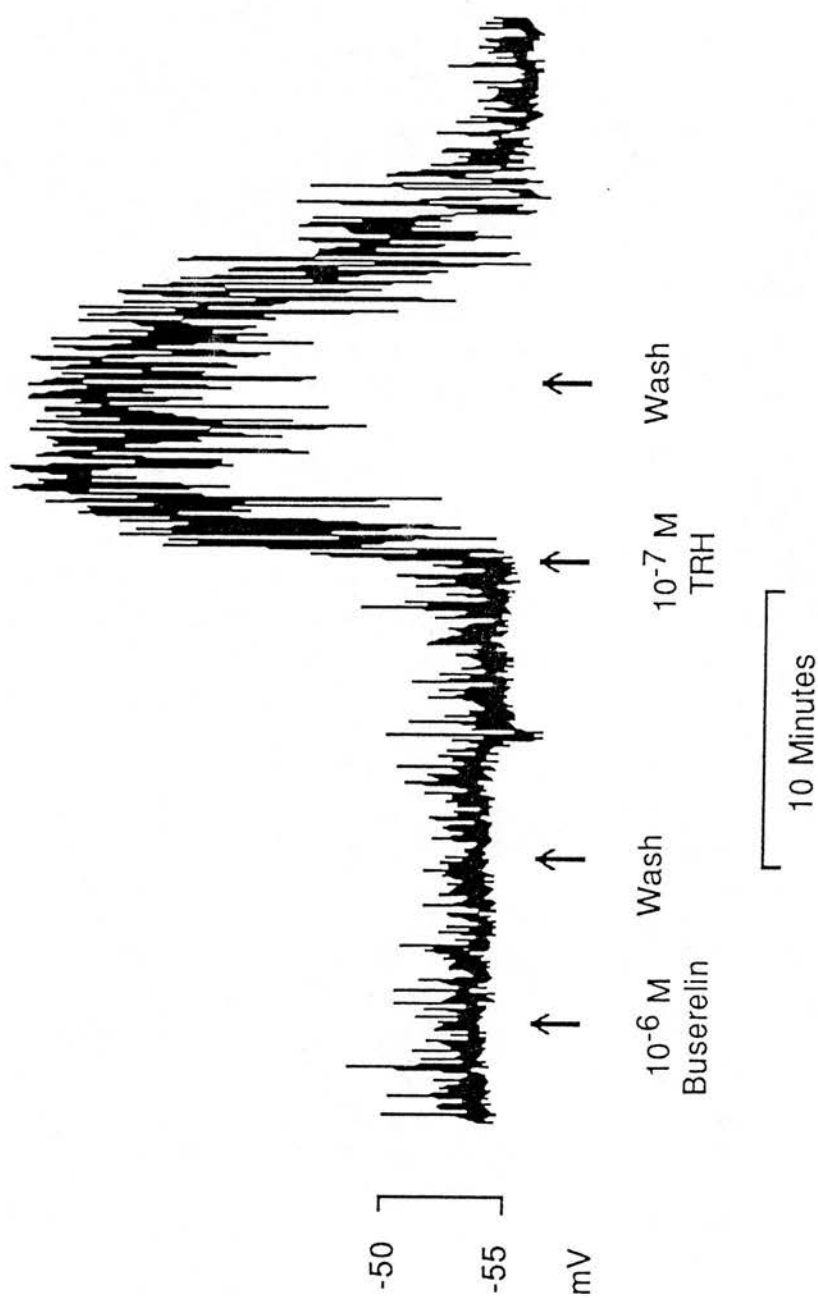
The addition of TRH produced a response in 70% of oocytes. An example of such an oocyte is shown in Figure 3.3, in which the application of the GnRH agonist busserelin (1 $\mu$ M) produced no response, but TRH (0.1 $\mu$ M) produced a response consisting of numerous membrane depolarisations which had a

**Figure 3.2** Inhibition of the GnRH agonist-induced response by a GnRH antagonist in a mRNA-injected oocyte. In (A), the arrow indicates the application of of the GnRH agonist (0.1 $\mu$ M in the presence of a GnRH antagonist (0.1 $\mu$ M) and (B) shows the application of the antagonist during a response elicited by the application of the GnRH agonist (0.1 $\mu$ M).





**Figure 3.3** A typical response to TRH after the injection of mRNA from the rat pituitary. The oocyte did not respond to 1 $\mu$ M GnRH agonist (Buserelin). A membrane depolarisation was produced by a subsequent application of TRH (0.1 $\mu$ M).



maximum amplitude of around 20mV. In such TRH responsive oocytes, TRH produced depolarising responses in a dose-dependent manner. An example of an oocyte showing this dose dependency is shown in figure 3.4, where the application of 10nM TRH produces individual depolarisations (maximum amplitude 20mV) and the application of 0.1μM TRH produces an initial continuous depolarisation of 31mV. The threshold for activation of a TRH response was around 1nM and the maximum response occurred at a concentration of about 1μM.

Repetitive application of TRH showed that desensitisation of the receptor may occur. Figure 3.5 illustrates a typical example of such an oocyte. The application of TRH (0.1μM) produced a very rapid continuous membrane depolarisation (within 10sec), which decayed over the next 20 minutes (Figure 3.5A). Application of GnRH or its agonist produced no response in this oocyte (Figure 3.5B). The oocyte did, however, remain responsive to TRH (0.1μM). Upon the second application of TRH the latency of the depolarisation was longer and the frequency of the depolarisations was smaller (Figure 3.5C). In other oocytes, where the interval between applications was shorter, the latency of the second response was much longer and the amplitude, in addition to the frequency of the depolarisations was markedly reduced.

The only known antagonist of the TRH receptor is the calmodulin inhibitor, chlordiazepoxide, which binds competitively to pituitary TRH receptors and shifts the dose-response curve to the right (Simasko & Horita, 1985; Drummond, A.H. 1985). In three cells the application of chlordiazepoxide produced an inhibition of the TRH response. The application of TRH (0.1μM) in the presence of chlordiazepoxide (0.2μM) produced no membrane response (Figure 3.6). After several minutes of wash a second application of TRH produced the usual depolarising response. Application of the GnRH antagonist had no effect on the TRH response.

A further interesting finding was, in the three oocytes tested, 1mM cadmium (known to block calcium channels) appeared to produce a facilitation of the response to TRH. This apparent facilitation is shown in figure 3.7, where the initial application of TRH produced individual depolarisations of 20mV the application of Cd<sup>2+</sup> in the continuous presence of TRH produced a sustained depolarisation of 25mV. Similar apparent facilitations were seen in the presence of similar concentrations of Mn<sup>2+</sup> and Ni<sup>2+</sup>.

**Figure 3.4** The induced response to TRH after injection of rat pituitary mRNA occurred in a dose-dependent manner. In (A) the response to 10nM TRH consisted of a smaller response to that elicited by a subsequent application of 0.1 $\mu$ M TRH. The dose-dependency of the response to TRH (from 10 oocytes) is shown in (B).

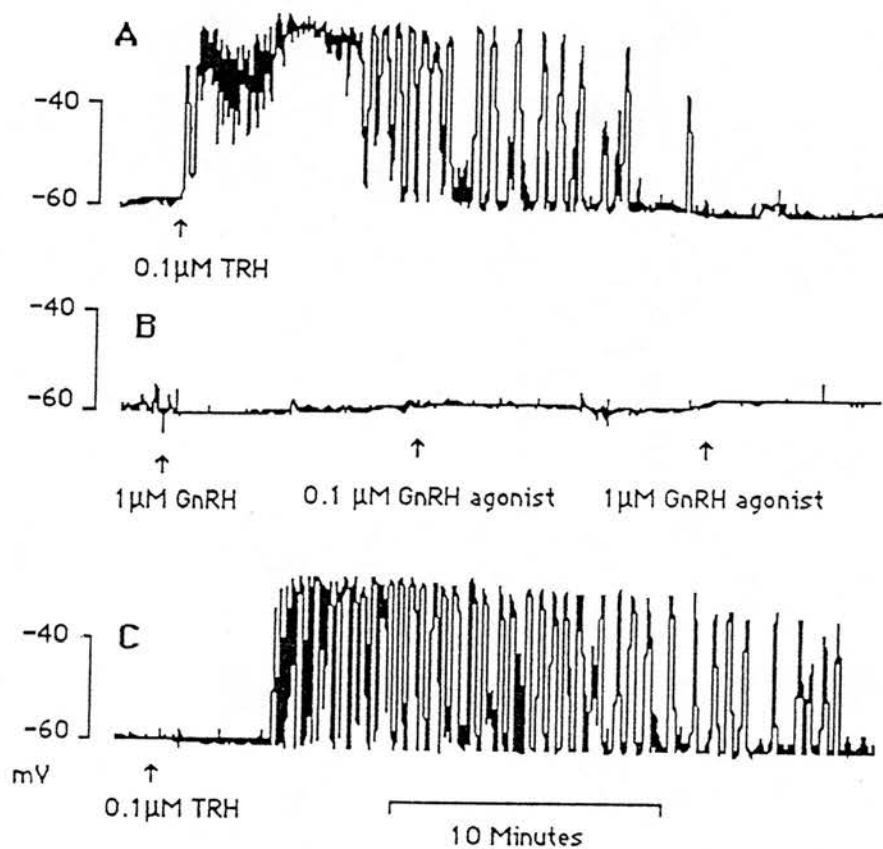
10 nM TRH

0.1  $\mu$ M TRH

-60  
mV  
-80

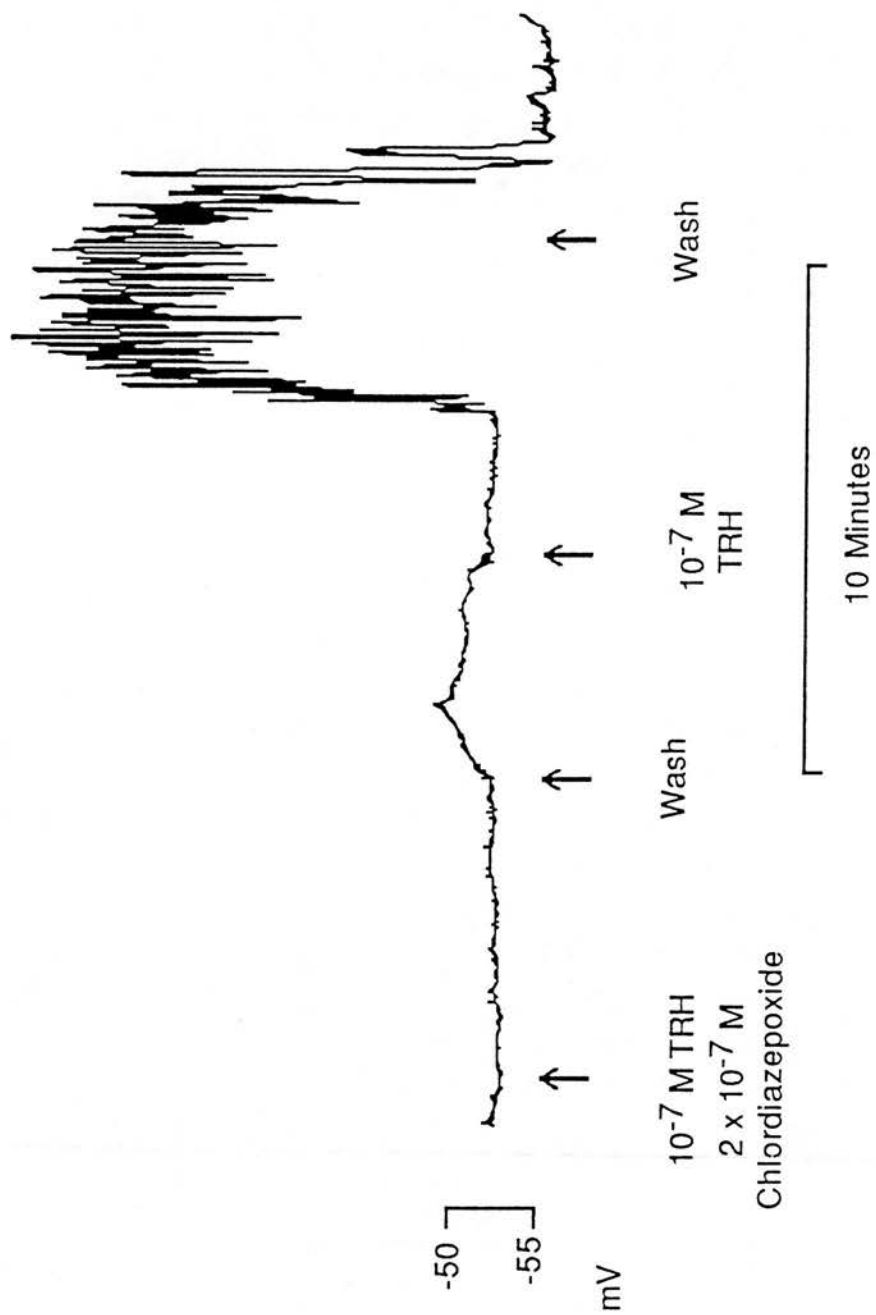
5 min

**Figure 3.5** Membrane potential changes induced by successive applications of TRH in an oocyte injected with poly (A)+ RNA . The oocyte responded to 0.1 $\mu$ M TRH (A), but did not respond to 1 $\mu$ M GnRH or 0.1 M GnRH agonist (B). The oocyte remained responsive to TRH (0.1 $\mu$ M), But the latency of the response was longer and the size of the response was reduced (C).

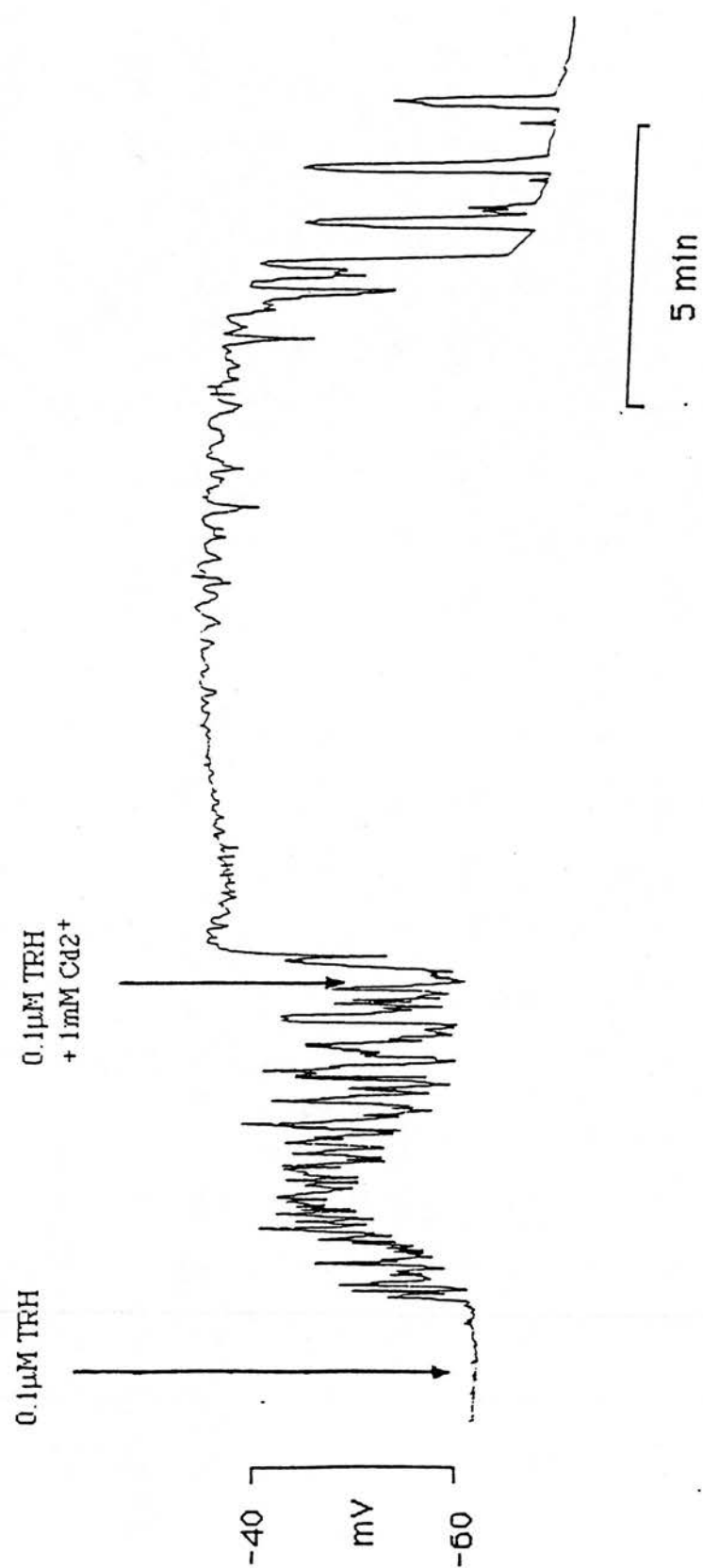


**Figure 3.6** The effect of the calmodulin inhibitor chlordiazepoxide. The mRNA-injected oocyte showed no response to TRH ( $0.1\mu\text{M}$ ) in the presence of chlordiazepoxide ( $0.2\mu\text{M}$ ). The responsiveness to TRH ( $0.1\mu\text{M}$ ) was regained after several minutes of washing to remove the chlordiazepoxide.





**Figure 3.7** The effect of the divalent cation cadmium ( $\text{Cd}^{2+}$ ) on the TRH response induced in a mRNA-injected oocyte. The response to TRH ( $0.1\mu\text{M}$ ) consists of individual depolarisations, which in the additional presence of  $\text{Cd}^{2+}$  ( $1\text{mM}$ ) becomes a sustained depolarisation.



A small proportion of the oocytes responded to both ligands, an example of such an oocyte is shown in figure 3.8). Obviously, in oocytes which are known to have some endogenous receptors (see section 1), it is important to carry out control experiments<sup>(n=11)</sup>, such a control experiment is shown in Figure 3.9). This oocyte was injected with sterile deionised water and then treated in the same way as the mRNA-injected oocytes. The application of the GnRH agonist and TRH produced no response. Therefore, the responses seen to GnRH and TRH are due to the functional expression of GnRH and TRH receptors.

## Discussion

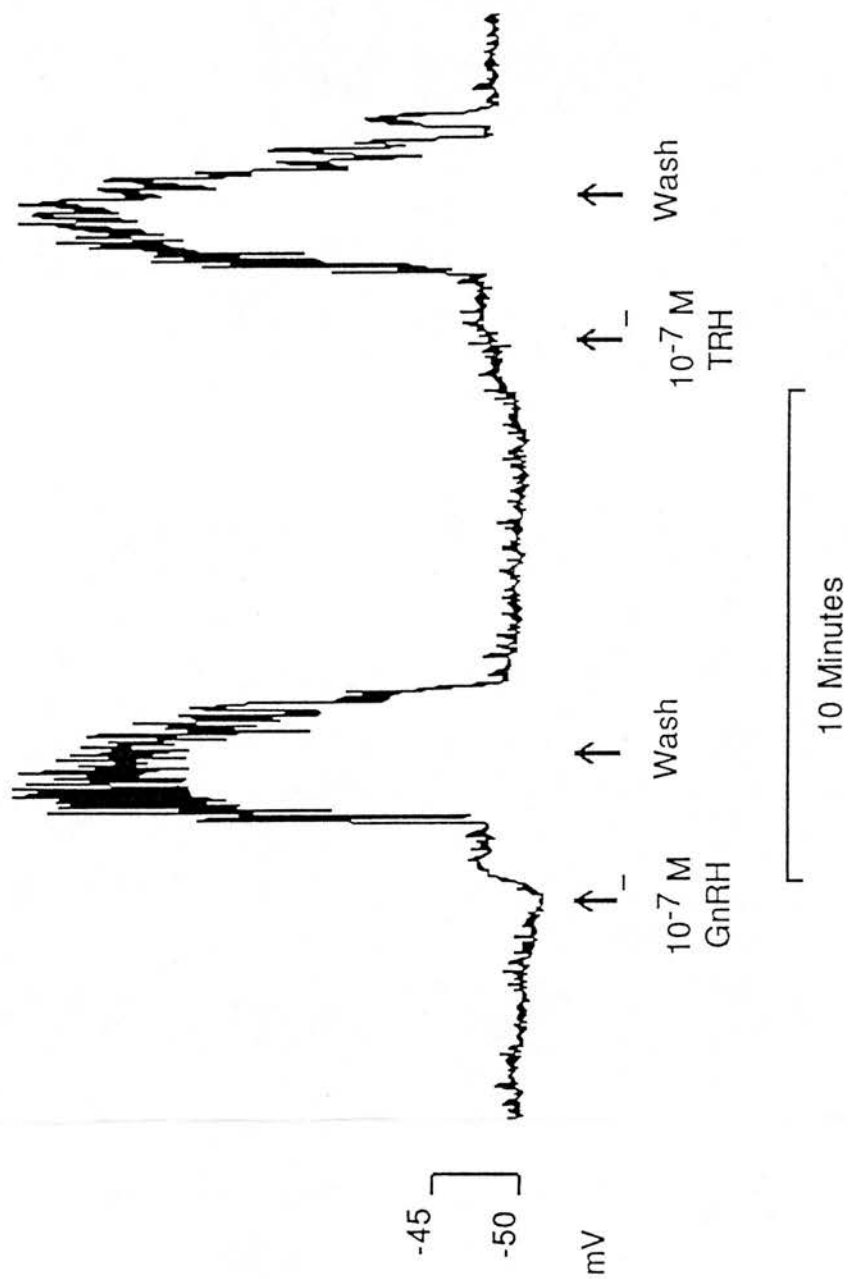
The present data show that poly (A)+ mRNA obtained from the rat pituitary gland encoding peptide receptors, including those for GnRH and TRH can be expressed in *Xenopus* oocytes.

This is the first report of the functional expression of the GnRH receptor. There are several findings which indicate that the expressed receptor is specific for GnRH. Firstly, control (water or uninjected) oocytes show no response to GnRH. Secondly, the membrane depolarisations produced by GnRH can also be elicited by a known GnRH agonist (buserelin) (Friedrich, Etztrodt, Becker *et al.* 1978). Thirdly, the response to GnRH or the agonist are inhibited by a GnRH receptor antagonist.

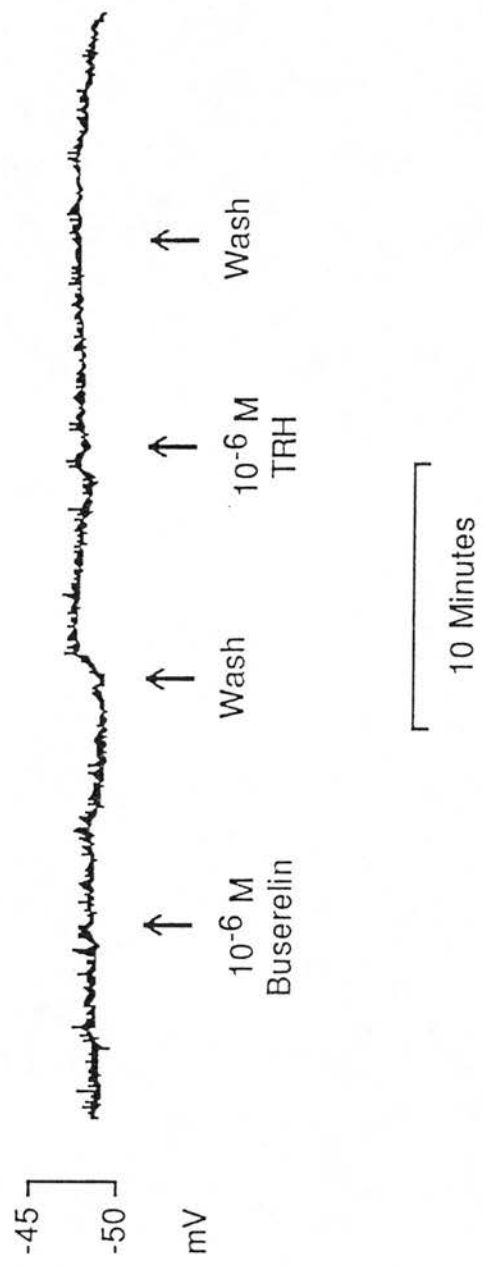
The GnRH receptor translated into the oocyte has some pharmacological kinetics which are similar to those of the native GnRH receptor in the pituitary gland. In the pituitary the GnRH agonist which was used in the present study was 100-fold more potent than GnRH in activating the GnRH receptor of the pituitary gland (Friedrich, Etztrodt, Becker *et al.* 1978). A not dissimilar difference in potency was seen in the current study (where 0.1 $\mu$ M agonist produced a much larger response, in both amplitude and frequency, than 1 $\mu$ M GnRH). Likewise, the action of GnRH or its agonist was inhibited by the GnRH receptor antagonist.

Oocytes expressing TRH receptors were seen more frequently than those expressing GnRH. This might be because the TRH receptor mRNA shows a greater stability in this system and that the TRH receptor mRNA is more abundant than the GnRH receptor mRNA in the pituitary. The dose

**Figure 3.8** An example of co-expression of GnRH and TRH receptors following injection of rat pituitary mRNA. The application of GnRH ( $0.1\mu\text{M}$ ) produces a large depolarisation which is followed, after several minutes of washing, by a typical depolarising response to TRH ( $0.1\mu\text{M}$ ).



**Figure 3.9** A control oocyte. An oocyte injected with 50ng of water instead of mRNA shows no response to either GnRH agonist (1 $\mu$ M) or to TRH (1 $\mu$ M).





dependency of the response to TRH was similar to that reported by Oron, Gillo, Straub & Gershengorn (1987)(1nM-1 $\mu$ M), who obtained their receptor mRNA from the TRH receptor cell-line GH3. The response to TRH was found to be blocked by the TRH antagonist chlordiazepoxide at a concentration of 0.2 $\mu$ M. A similar dependency on chlordiazepoxide was demonstrated in oocytes expressing TRH receptors from the GH3 cell line (Oron, Gillo, Straub & Gershengorn, 1987)

The preliminary finding of these experiments, that the divalent cations Cd<sup>2+</sup>, Mn<sup>2+</sup> and Ni<sup>2+</sup> produced a facilitation of the response to TRH was recently studied in a paper by Miledi, Parker and Woodward (1989). In this study they showed that the application of divalent ions such as Cd<sup>2+</sup>, Ni<sup>2+</sup>, and Mn<sup>2+</sup> (in the concentration range 1 $\mu$ M-1mM), could produce slow oscillatory membrane currents. In discussion they concluded that these oscillatory currents are due to an interaction with the oocyte membrane which causes a rise in the intracellular concentration of inositol phosphates, because intracellular injection of inositol 1,4,5-trisphosphate produced similar currents and attenuated the response to the divalent cations. This rise in inositol phosphates is thought to cause a rise in intracellular calcium and hence an activation of calcium-dependent chloride channels. Hence the apparent facilitation of the oscillations seen in the oocytes expressing TRH receptors could due to an indirect effect on the membrane. However, the possibility remains that by blocking the plasma membrane calcium pumps the extrusion of calcium is prevented and a large rise in the internal concentration of calcium would occur which would explain the apparent facilitation of the TRH response.

Some desensitisation was observed in both GnRH and TRH responsiveness. In oocytes continuously exposed to GnRH, responses to GnRH were observed to continue for periods in excess of 15 minutes, although the absolute duration of the response was not studied in detail, the responses appeared to decline and stop after approximately 20 minutes, although considerable variation in the duration of the response was seen in the oocytes. Desensitisation is a well studied phenomenon in the brain. In the pituitary, it is well established that exposure of gonadotropes to GnRH results in receptor regulation (Zilberstein, Zakut & Aor, 1983; Conn, Rogers & Seay, 1984) and in the regulation of cellular responses (De koning, van Deiten & van Rees, 1978; Badger, Loughlin & Naddaff, 1983). Some of this desensitisation is due to receptor internalization and is measurable after 20 minutes (see Conn, 1986) and would

seem to be comparable to the desensitisation seen in this study. Such a finding suggests that the desensitization lies with the receptor and not with the responsiveness of the oocyte.

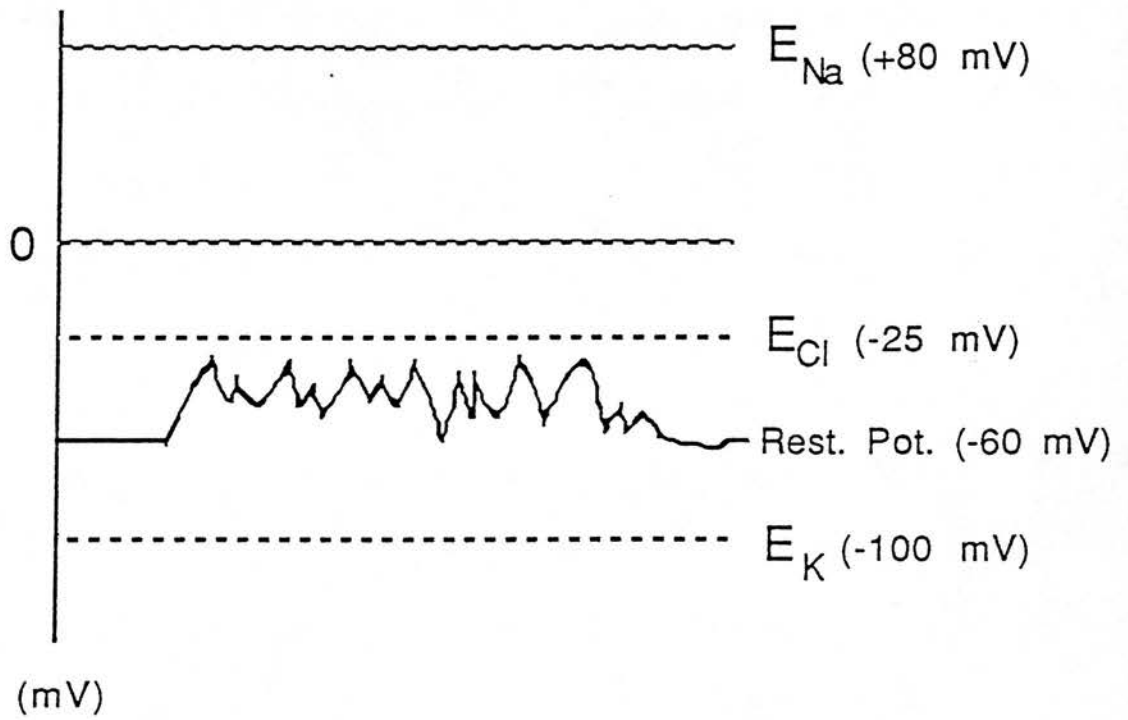
Similarly the response to TRH in the continued presence of the agonist declined over about 30 minutes and successive applications of TRH elicited smaller responses. Such a desensitization may be expected from TRH receptor since treatment of rats with TRH has been shown to down regulate receptors in the brain (Ogawa, Mizuno, Nukina *et al* , 1983; Simasko & Horita, 1984) and in the pituitary (Banenyee & Prasad, 1982).

The latency of the response to GnRH or TRH varied from oocyte to oocyte, the range being between 1 and 5 minutes. However, it was apparent that the latency was longer when a lower concentration of ligand was applied. A similar effect has been shown recently in oocytes expressing TRH receptors ( Straub, Oron, Gillo *et al.*, 1989) where the duration of the latency was shown to be inversely related to the dose of TRH and to the amplitude of the response. This relationship was shown to be due to the number of receptors expressed. It is interesting to note that in oocytes expressing exogenous acetylcholine (ACh) receptors the latency of the response is slower than that of the ACh receptors which are endogenous to the oocyte (Oron, Gillo & Gershengorn, 1988). This suggests that the relatively long latency seen in the response of expressed receptors (in the order of minutes) is due to some rate limiting step which does not appear to be endogenous to the oocyte.

Whilst the use of conventional intracellular techniques is convenient for an initial study of the feasibility of expression of receptors it has certain limitations. In particular it is not possible to make comparisons between the response seen in oocytes with different resting membrane potentials. However the maximum depolarisation seen was -30mV, from a resting membrane potential of around -60mV. This value may give an indication of the ion movement responsible for the change in the membrane potential. The equilibrium potentials for different ions (when the net driving force for that ion is zero) are shown in Figure 3.10. Therefore, if the response to GnRH consisted of an opening of K<sup>+</sup> channels, as has been shown to occur in pituitary gonadotropes (Croxtan, Ben-Jonathan & Armstrong, 1988) the membrane would hyperpolarise to around -100mV. As the membrane depolarises to around -30 mV in these experiments, it would seem likely that

**Figure 3.10** A diagram of the equilibrium potentials for  $K^+$ ,  $Na^+$  and  $Cl^-$  in *Xenopus* oocytes (dotted lines). The filled line is a diagrammatic representation of the resting membrane potential and the effect of the addition of GnRH or TRH.

Membrane Potential -



the response to TRH and GnRH is mediated by an opening of  $\text{Cl}^-$  channels (whose equilibrium potential is around  $-25 \text{ mV}$ ). However, the possibility remains that the response to these ligands could activate both  $\text{Na}^+$  and  $\text{K}^+$  channels, which could result in a similar depolarisation. Further study of the ionic dependency of the response, using the two-electrode voltage-clamp technique, is necessary.

## SECTION 4

### Responses elicited from *Xenopus* oocytes injected with rat anterior pituitary mRNA measured using voltage-clamp recording techniques.

The aim of this section is to give an account of the responses elicited by the application of various hypothalamic releasing factors and other hormones and peptides, known to play a role in the control of anterior pituitary hormone release, to *Xenopus laevis* oocytes which have been injected with rat anterior pituitary gland mRNA. Cellular responses were monitored using the two-electrode voltage-clamp technique which is detailed in the methods section.

## RESULTS

Following the injection of rat anterior gland poly (A)+ mRNA the *Xenopus* oocytes became responsive to a number of releasing hormones from the hypothalamus. This responsiveness appears to be due to the expression of rat anterior pituitary receptors, as these responses did not occur in control oocytes which were not injected with rat anterior brain mRNA.

### The expression of GnRH and TRH receptors

In a study of 50 oocytes injected with poly (A)+ mRNA, the expression of GnRH and TRH receptors was studied intensively. Typical responses elicited by GnRH and TRH, in oocytes clamped at -60 mV, are shown in Figure 4.1 A and B. In Figure 4.1 A the application of GnRH (1 $\mu$ M) to the external medium produced a typical response which consisted of a slow prolonged inward current with superimposed fluctuations. In this oocyte the response to GnRH had an amplitude of 50 nA and occurred with a latency of 1.4 min. Such a response was seen from 64% of the oocytes studied. The mean amplitude of the response to GnRH (1 $\mu$ M) was  $187 \pm 82$  nA and the latency was  $56 \pm 32$  sec.

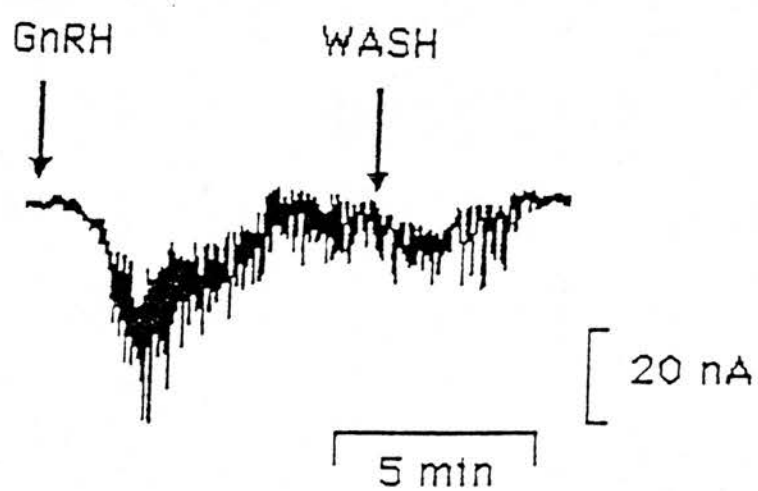
In Figure 4.1 B the application of TRH in a different oocyte produced a similar inward current response to that seen for GnRH but the latency was generally shorter and the amplitude larger. In Figure 4.1 B the amplitude of the response to 1 $\mu$ M TRH was 140 nA and the latency was 40 sec. From the 50 oocytes studied 84% of the oocytes acquired responsiveness to TRH, the mean amplitude being  $272 \pm 156$  nA and the latency  $26 \pm 19$  sec following exposure to 1 $\mu$ M TRH. The expression of TRH receptors was seen in oocytes which also expressed GnRH receptors (in 48% of oocytes tested) but the TRH response

**Figure 4.1** Membrane currents induced by the application of GnRH and TRH in mRNA-injected oocytes.

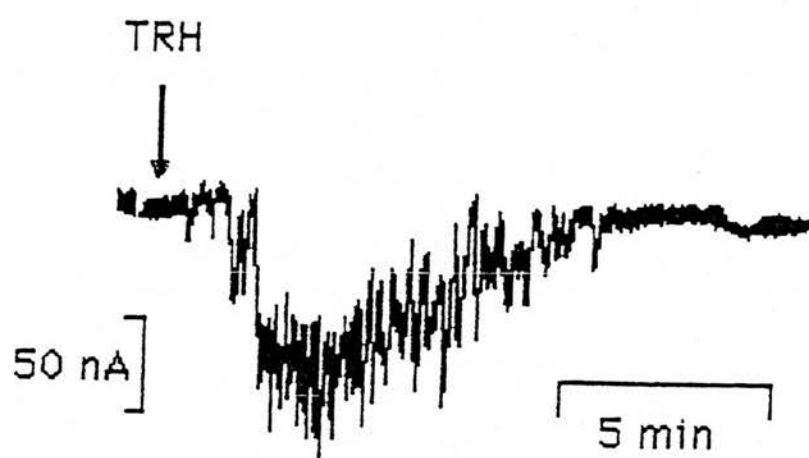
The oocyte was injected with approximately 50 ng of poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands and incubated for 4 days. Whole-cell current records were obtained using two-electrode voltage-clamp techniques. In (A) Current fluctuations superimposed on a transient inward current were evoked by the application of 1  $\mu$ M GnRH (gonadotropin-releasing hormone). In (B) the application of TRH (1 $\mu$ M) produced a transient inward current with superimposed current fluctuations.

In this and all other figures the holding potential was -60 mV, inward currents are shown as downward deflections and the point of addition of peptide or washout with standard solution by perfusion of the experimental chamber are indicated by arrows.

**A**



**B**





could also be elicited in oocytes in which there was no response to GnRH (36% of the oocytes studied). This differential expression provides evidence that rat anterior pituitary mRNA encodes separate receptors for GnRH and TRH. Control oocytes, those either uninjected or injected with 50nl of water (which was used as a solvent for mRNA) were unresponsive to GnRH or TRH.

### **The expression of Dopamine receptors**

The action of dopamine was studied in oocytes injected with poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary. An example of the effect of the application of dopamine to oocytes which had been injected with mRNA is shown in Figure 4.2 A. In this oocyte the application of dopamine (1mM) produced no change in the membrane current. The application of acetylcholine (1 $\mu$ M) induced inward current fluctuations of around 10nA. Such current fluctuations in response to ACh also occur in uninjected oocytes and are due to the presence of endogenous ACh receptors in the plasma membrane of the oocyte (Dascal & Landau, 1980). It seems reasonable to suppose that poly(A)<sup>+</sup> mRNA obtained from the rat anterior pituitary gland should encode the many types of receptors which exist in the pituitary gland, including those for dopamine as well as the above mentioned TRH and GnRH receptors. As dopamine is known to have an inhibitory action in the pituitary (on the release of prolactin from thyrotroph cells), the effect of dopamine on the response to TRH was studied. In such oocytes (n=5), an example of which is shown in Figure 4.2 B, the application of TRH (0.1  $\mu$ M) in the presence of dopamine (1 $\mu$ M) produces only a small membrane response, which increases briefly on washing the oocyte (possibly because the inhibitory effect of dopamine washes off faster than the stimulatory effect of TRH on the TRH receptors). Following extensive washing of the oocyte the subsequent application of TRH (0.1 $\mu$ M) induces the usual large inward current response. Thus it seems apparent that dopamine had an inhibitory action on the response to TRH.

Interactions have also been reported between dopamine and serotonin receptors. To study the effect of dopamine on the response to serotonin, serotonin receptors were expressed in *Xenopus* oocytes by injecting a mRNA made from cloned cDNA encoding the 5-HT<sub>1c</sub>-receptor (courtesy of Dr. D. Julius; Julius, MacDermott, Axel *et al.*, 1988). In oocytes injected with rat anterior pituitary mRNA, or uninjected, the application of serotonin (1 $\mu$ M) produces no change in the membrane current (Figure 4.3). In an oocyte which has been injected with mRNA encoding the serotonin (5-HT<sub>1c</sub>) receptor, an example of which is shown in Figure 4.3A the addition of serotonin (1 $\mu$ M) elicited a typically large

**Figure 4.2** Membrane currents induced by the application of dopamine and TRH in mRNA-injected oocytes.

In (A) an oocyte injected with rat anterior pituitary gland mRNA produced no current response to dopamine (1mM) or serotonin (1 $\mu$ M). The application of acetylcholine (ACh) produces a very small, fluctuating inward current response.

In (B) The application of TRH (0.1 $\mu$ M) in the presence of dopamine (1 $\mu$ M) produces only a small inward current. Following extensive washing, a second challenge of TRH (0.1  $\mu$ M) produces a large inward current response.

A

CONTROL

1 mM  
Dopamine1  $\mu$ M  
ACh1  $\mu$ M  
Serotonin

50 nA

5 minutes

B

0.1  $\mu$ M TRH  
1  $\mu$ M Dopamine wash0.1  $\mu$ M TRH

200 nA

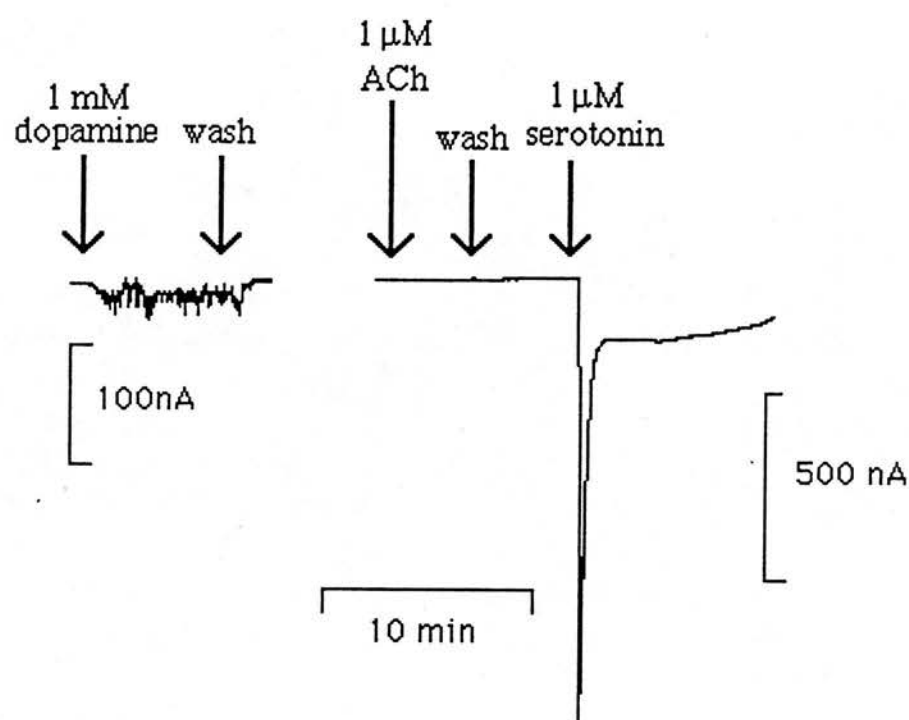
5 minutes

**Figure 4.3** Membrane currents induced by the application of dopamine and serotonin in oocytes injected with mRNA encoding the serotonin 5-HT 1c receptor.

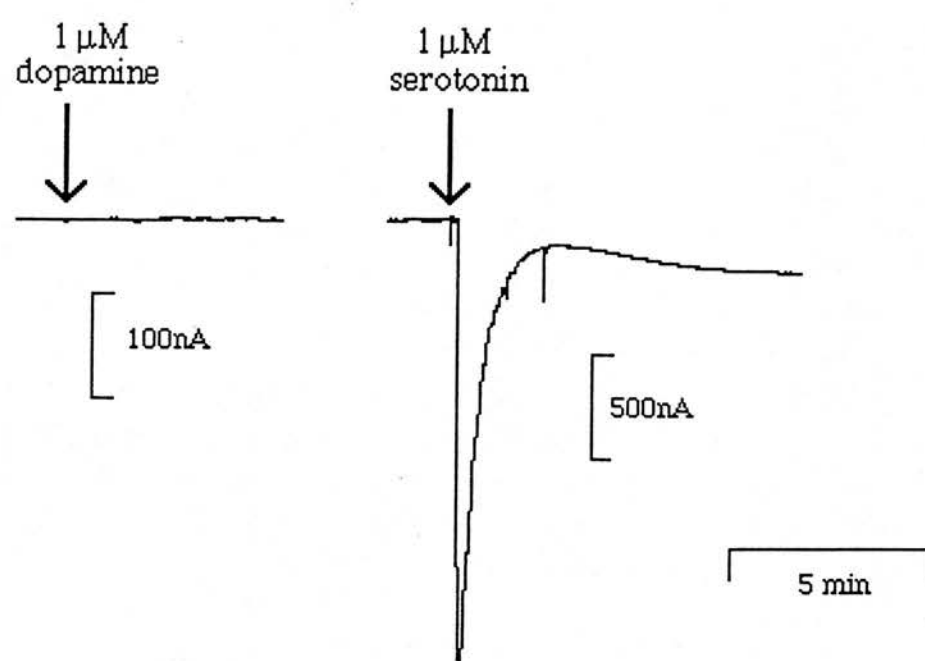
In (A) the application of dopamine (1mM) to an oocyte expressing serotonin receptors produces an oscillatory inward current. The application of acetylcholine (ACh, 1 $\mu$ M) produces no change in the membrane current. The addition of serotonin (5-HT, 1 $\mu$ M) produced a large, fast transient response followed by a slowly inactivating inward current.

In (B) the application of dopamine (1 $\mu$ M) to an oocyte expressing serotonin receptors produces no change in the membrane current. The addition of serotonin (5-HT, 1 $\mu$ M) produced a large, fast transient response followed by a slow, sustained inward current.

A



B



serotonin response consisting of a fast transient inward current followed by a slow inward current. This oocyte did not respond to ACh (it has been reported that the number of endogenous ACh receptors varies from oocyte to oocyte). Interestingly, the oocyte also responded to 1 mM dopamine to generate oscillatory inward currents of 40nA. Such a response to dopamine was observed only in oocytes which expressed serotonin receptors and only when dopamine was added at a high concentration (1mM). When the concentration of dopamine was low, 1  $\mu$ M, no inward current was elicited in oocytes in which serotonin receptors were expressed, as shown in Figure 4.3 B. Hence dopamine appears to activate the 5-HT 1c receptor, presumably by a direct activation of the receptor. In these oocytes expressing the 5-HT receptor, the application of serotonin in the presence of dopamine appeared to have no inhibitory effect on the serotonin response (presumably because no dopamine receptors were expressed in the membrane of the oocyte).

#### **A study of other hypothalamic anterior pituitary hormone-releasing hormones.**

As mentioned in the introduction to this section there are many hypothalamic releasing hormones which exert an effect on pituitary cells, presumably by activating specific receptors. In several oocytes, which had been injected with rat anterior pituitary mRNA, the application of corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GRH) and somatostatin (1 $\mu$ M) produced no change in the membrane current. This however does not necessarily mean that the receptor has not been expressed. As with the dopamine receptor, the effect of stimulation of the receptor may be different to that induced by the activation of the GnRH and TRH receptors and not involve the opening of ion channels.

#### **The expression of Melatonin and Neuropeptide Y (NPY) receptors**

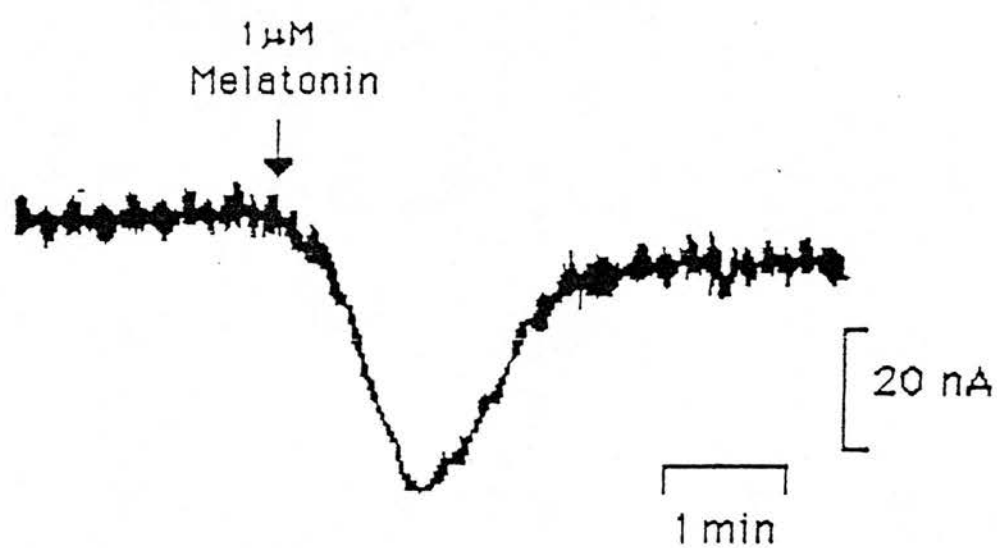
A study of the effect of the application of the pineal gland hormone melatonin from three oocytes injected with rat anterior pituitary gland mRNA found that the application of melatonin (1 $\mu$ M) typically produced the response shown in Figure 4.4 A. In this oocyte the addition of melatonin to the bathing solution produced a smooth, transient inward current, the amplitude of which was 40 nA and the latency 11 seconds, the current peaked after 60 sec and then inactivated. The mean amplitude of the response to melatonin (1 $\mu$ M) was  $21.3 \pm 16.3$  nA and the latency was  $12.0 \pm 2.7$  sec. In all the oocytes studied the

**Figure 4.4** Membrane currents induced by the application of melatonin and neuropeptide Y (NPY) in mRNA-injected oocytes.

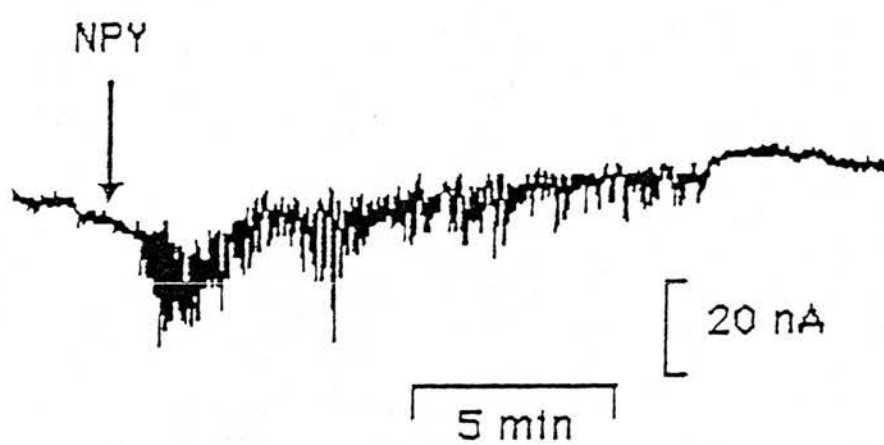
In (A) the application of melatonin ( $1\mu\text{M}$ ) to an oocyte injected with mRNA from the rat anterior pituitary gland and clamped at  $-60\text{mV}$ , produces a smooth, sustained inward current which rapidly desensitized.

In (B) the application of NPY ( $1\mu\text{M}$ ) induced a transient inward current with superimposed current fluctuations.

A



B





response to melatonin was a smooth inward current with no current fluctuations. As with the GnRH and TRH receptor expression, control oocytes showed no responsiveness to melatonin.

In a preliminary study of the effect of the addition of neuropeptide Y (NPY) to oocytes injected with rat anterior pituitary gland mRNA, NPY (1 $\mu$ M) produced a slow prolonged inward current with superimposed fluctuations in two of the three oocytes studied. An example of this response is shown in Figure 4.4 B where the application of NPY (1 $\mu$ M) produced a typical inward current response of 20 nA with a latency of 60 seconds. In the other oocyte studied the application of NPY elicited a response of 10 nA with a latency of 150 seconds. Control oocytes showed no response to the application of NPY. The response is similar to that elicited following the application of GnRH, but the response would seem to be due to a receptor which is separate from the GnRH receptor, as found from an observation that there was an <sup>apparent</sup> facilitatory effect of NPY on the GnRH response. This effect is shown in Figure 4.5, where the application of GnRH produced the usual inward current fluctuations, the subsequent addition of NPY (1 $\mu$ M) in the continued presence of the GnRH produced a smooth inward current of 100 nA superimposed on the inward current fluctuations.

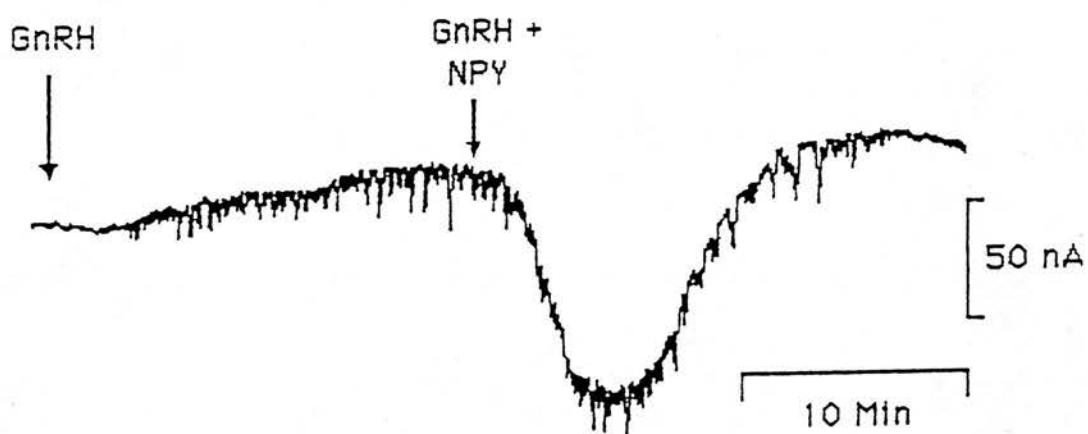
## Discussion

### Expression of GnRH and TRH receptors

The above results indicate that the injection of rat anterior pituitary mRNA causes the expression of receptors for both GnRH and TRH. These receptors appear to be expressed independently of each other, which provides some evidence that two distinct receptors are being expressed. Activation of either receptor causes the production of a smooth inward current with superimposed inward current fluctuations. Such a current response is typical of the *Xenopus* oocyte expressing exogenous receptors such as angiotensin II and arginine vasopressin (Meyerhoff, Morley, Schwartz *et al.*, 1988) and the expression of TRH receptors in the same study was shown to be similar to that shown here. Such a response in the *Xenopus* oocyte, which occurs with a relatively long latency, is thought to be due to the activation of an endogenous Cl<sup>-</sup> channel subsequent to the activation of a G-protein which is linked to the inositol trisphosphate pathway (Dascal, 1987). Recently it has been reported that Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels in *Xenopus* oocytes are activated by direct injection of activated  $\alpha$ -subunit of the PTX-sensitive G-protein (Go) by mobilizing Ca<sup>2+</sup>

**Figure 4.5** Membrane currents induced by the application of GnRH and NPY in the same mRNA-injected oocyte.

In this oocyte the application of GnRH (1 $\mu$ M) produces a fluctuating inward current response. The subsequent application of NPY (1 $\mu$ M) in the continued presence of GnRH produces an inactivating inward current response and no change in the amplitude or frequency of the fluctuating response.



from intracellular inositol 1,4,5-trisphosphate (IP3)-sensitive stores (Moriarty, Padwell, Carty *et al.*, 1990). The many steps before the activation of the Cl-channel are thought to be responsible for the long latency observed in such responses.

A study of these two receptors is of particular interest because they control a number of vital functions in the anterior pituitary gland. GnRH stimulates the secretion of LH and FSH by gonadotropes. FSH stimulates ovarian follicular growth. LH stimulates the interstitial cells of both the ovary and testes. TRH has more diverse actions in the pituitary gland. TRH stimulates the release of prolactin (PRL) from lactotroph cells and the release of thyroid stimulating hormone (TSH) from thyrotroph cells. PRL induces mammary gland growth and milk production following delivery of an infant. In rodents PRL affects gonadal function and helps maintain the structure and function of accessory sex organs. TSH stimulates secretion of the hormones thyroxine and triiodothyroxine by the thyroid gland. A more detailed study of these receptor responses in *Xenopus* oocytes is presented in Sections 5 and 6.

### **Inhibitory action of dopamine on the TRH response**

The results show that dopamine inhibited the response to TRH. It seems likely that the oocyte injected with poly(A)<sup>+</sup> mRNA from the anterior pituitary gland would express both dopamine and TRH receptors as dopamine receptors are present in abundance in the anterior pituitary gland. Therefore, a reasonable conclusion from the above experiments is that dopamine inhibited the TRH response through the dopamine receptor. Such a dopaminergic inhibition of the TRH has been reported by Schofield (1983) who showed that dopamine antagonized the increase in intracellular levels of Ca<sup>2+</sup> elicited by TRH in anterior pituitary cells. In addition, dopamine has been also reported to inhibit release of prolactin from anterior pituitary cells in response to angiotensin II and to inhibit the production of inositol trisphosphate induced by angiotensin II (Enjalbert, Sladeczek, Guillon *et al.*, 1986). Interestingly, an inhibitory effect of dopamine on gonadotropin release has been reported in many teleost fishes such as carp, goldfish, eel and catfish (for review see Peter, Chang, Nahorniak *et al.*, 1986 and De Leeuw, Goos & Van Oordt, 1987). This dopaminergic inhibition of gonadotropin release is mediated by dopamine receptors similar to the mammalian D<sub>2</sub> type (Chang, Peter, Nahorniak & Sokolowska, 1984; Omeljaniuk, Shih & Peter, 1987; Omeljaniuk, Tonon & Peter, 1989). In the

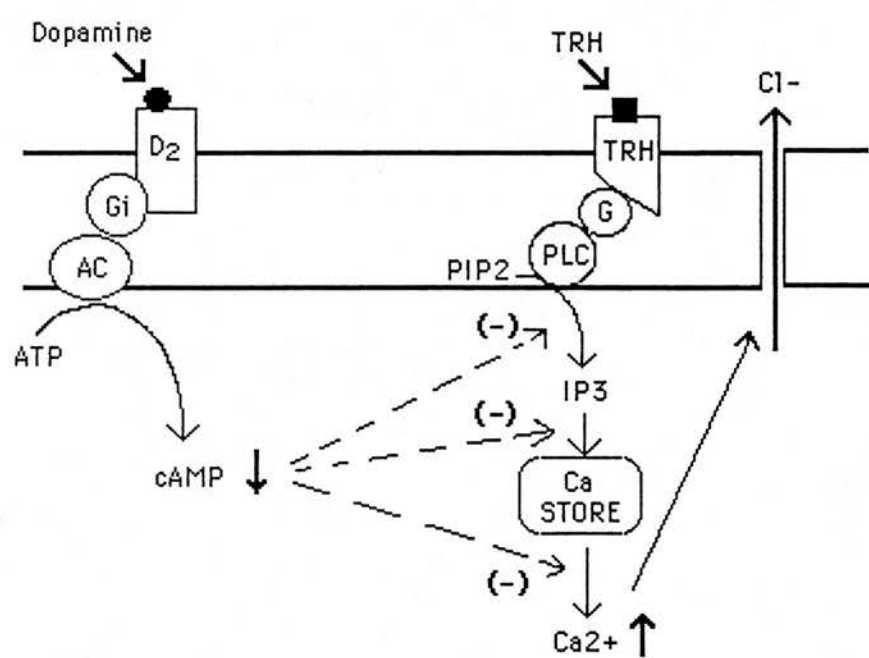
present study the effect of dopamine administration on the response to GnRH in *Xenopus* oocytes was studied, but no conclusive data have yet been obtained. From the results of the effect on the TRH response we can tentatively conclude that the dopamine receptor expressed has an inhibitory action. There is a possibility that the dopamine has a direct effect on the TRH receptor. The action of dopamine could be better studied once the cloning of the TRH receptor has been accomplished. Assuming a dopamine receptor is expressed and that it has an inhibitory action suggests that it is the dopamine D<sub>2</sub> receptor, which has recently been cloned and sequenced (Bunzow, Van Toll, Grandy *et al.*, 1988). Indeed, this assumption that rat anterior pituitary mRNA contains a dopamine D<sub>2</sub> receptor seems reasonable because a complementary DNA library, constructed from the mRNA used in this study, has been shown to contain the dopamine D<sub>2</sub> receptor (Eidne, Taylor, Zabavnik *et al.*, 1989).

The question which now arises is how the dopamine D<sub>2</sub> receptor causes its effect on the TRH response. In prolactinoma cells activation of the dopamine D<sub>2</sub> receptor has been demonstrated to be coupled to the inhibition of adenylate cyclase (De Camilli, Macconi & Spada, 1979). In purified lactotropes, a marked decrease in the level of cAMP was found to occur within 1 min of dopamine application, correlating with a reduced rate of prolactin secretion (Swennen & Denef, 1982). Thus the D<sub>2</sub> receptor inhibits adenylate cyclase to reduce the production of cAMP. Further light was thrown onto this subject by experiments studying the effect of dopamine on Ca<sup>2+</sup> mobilization from intracellular stores in lactotrophs. It was found that the rapid calcium transients induced by TRH in a Ca<sup>2+</sup>-free medium (where the sole source of Ca<sup>2+</sup> is the IP<sub>3</sub>-sensitive intracellular store) are inhibited by dopamine (Malgaroli, Vallar, Reza Elahi *et al.*, 1987). Hence IP<sub>3</sub>-induced Ca<sup>2+</sup> release operates under the permissive control of cAMP as a consequence of adenylate cyclase inhibition. A model of the possible events leading to an inhibition of IP<sub>3</sub> induced Ca<sup>2+</sup> release which occurs during the inhibition of the TRH response by dopamine is shown in Figure 4.6.

### **Activation of the serotonin receptor by dopamine**

In addition to this inhibitory effect on the TRH response, these experiments have revealed that dopamine appears to activate serotonin receptors. Serotonin receptors have been expressed in *Xenopus* oocytes using exogenous mRNAs derived from rat brain (Gundersen, Miledi & Parker., 1983a; Parker, Sumikawa & Miledi, 1985; Sakai, Kimura & Okamoto, 1986; Nomura, Kaneko, Kato *et al.*, 1987) in these studies responses to serotonin consisted of

**Figure 4.6** A model of the events taking place in the *Xenopus* oocyte membrane which cause dopamine to have an inhibitory action on the TRH response. In this diagram the following abbreviations have been used: D2 - Dopamine receptor type D2, Gi - an inhibitory G-protein, AC - adenylate cyclase, cAMP - cyclic adenosine mono phosphate, G - G-protein, PLC - phospholipase-C, PIP2 - phosphoinositol bisphosphate and IP3 - inositol trisphosphate.



a fast transient inward current, similar to that seen in the present study . Our results show that the application of dopamine to oocytes, either uninjected or injected with rat anterior pituitary mRNA, does not produce a membrane response but that in oocytes injected with mRNA encoding only the 5-HT receptor the application of a high concentration of dopamine (1mM) induces a small oscillatory inward current. Hence dopamine would appear to activate the serotonin receptor. Indeed, dopamine is known to activate 5-HT receptors with a relatively lower potency than 5-HT itself, about 100-times weaker (Neijt, Henk, Vijverberg *et al.* 1986; Neijt, Te Duits, Vijverberg *et al.* 1988; Peters & Usherwood 1984). This finding produces a possible explanation for the findings of Sumikawa, Parker & Miledi (1984), who reported an inward membrane current superimposed with an oscillatory current in response to dopamine (1mM) in *Xenopus* oocytes injected with rat brain mRNA. While it is possible that this response could be due to the activation of a different subtype of the dopamine receptor, which produces a stimulatory effect, their dopamine response was inhibited by a serotonin receptor antagonist methysergide (1  $\mu$ M). This suggests that the response to dopamine in their study was due to the activation of serotonin receptors. As mRNA from rat brain would also encode the serotonin receptor, the possibility that this response was due to the activation of 5-HT receptors by <sup>dopamine</sup> can not be excluded.

### Expression of melatonin receptors

Melatonin is produced by pinealocytes of the pineal gland and it has been suggested that it plays a role in the hypothalamic-pituitary gonadal axis (Reiter, 1978). Indeed oral administration of melatonin for a month in humans has been shown to cause a small reduction in serum LH (Nordung & Lerner, 1977). Despite large numbers of observations on the endocrine and non-endocrine effects of the pineal gland, little is known about how melatonin acts. Melatonin receptors have been sought in a variety of tissues but data on their existence and location is meagre. From the results presented here melatonin produces a membrane response distinct from that induced by GnRH and TRH, which suggests that a separate receptor is being activated. The response occurs with a latency of ~10 sec, much shorter than that seen for the GnRH and TRH responses and indeed any response which involves the activation of second messenger systems (Dascal, 1987). The response profile resembles that of the GABA<sub>A</sub> receptor expressed in *Xenopus* oocytes ( Parker, Sumikawa & Miledi, 1985), in which the receptor is directly linked to the channel responsible for the



response. Thus the melatonin receptor may be directly linked to a receptor-operated ion channel or the receptor itself may be an ion channel.

### **The induced responsiveness to Neuropeptide Y (NPY)**

Since the discovery of NPY in 1982 increasing evidence suggests that it modulates the secretion of reproductive hormones from the hypothalamus and pituitary gland (see McDonald, 1988, for review). NPY is normally present in high concentration in the pituitary portal blood system in rats (McDonald, Koenig, Gibbs *et al.*, 1987). The results of the present study show that NPY has a similar effect to GnRH in *Xenopus* oocytes injected with rat anterior pituitary mRNA, causing an oscillatory inward current response. This finding is not altogether surprising as NPY has been shown to directly stimulate LH and FSH secretion from the anterior pituitary gland *in vitro* (McDonald, Lumpkin, Samson & McCann, 1985). The finding that NPY increases and modulates the response to GnRH is also in keeping with the finding that NPY augments the release of LH by GnRH (Crowley, Hassad & Kalra, 1987). The findings of the present study are merely preliminary and the expression of the NPY receptor not unequivocally proved, but this area may reveal useful information about the properties of NPY in the future.

## SECTION 5

### Investigation of the response elicited by the activation of exogenous GnRH receptors using the two-electrode voltage-clamp technique

The results presented in this section form an investigation of the GnRH response expressed in *Xenopus* oocytes by the expression of rat anterior pituitary poly (A)+ mRNA . Included are data which reveal the signal-transduction pathway and the effector system. Preliminary attempts at the isolation of a clone which encodes the GnRH receptor are reported.

#### Current Responses induced by GnRH

Uninjected or water-injected *Xenopus* oocytes showed no response to GnRH or buserelin (an agonist of GnRH receptors) (not illustrated). Responses were observed in oocytes injected with poly(A)+-mRNA isolated from rat anterior pituitary glands following the application of GnRH (Fig. 5.1) . In the oocyte shown in Figure 5.1, the application of GnRH (1  $\mu$ M) to the external solution induced an inward current, the GnRH-induced response appearing with a latency of approximately 50 sec in this oocyte. To reduce the effect of desensitization of receptors, the peptide was washed from the bathing medium as soon as the maximum response was observed. A typical response consisted of a slow, prolonged inward current with superimposed fluctuations. The response reached its peak about 260 sec after the application of GnRH. Current fluctuations became less frequent and smaller in size on washing out the peptide. In this study, the total number of successful recordings of oocytes which were tested with GnRH was 50. Thirty two oocytes (64%) responded to GnRH. The latency and the time-to-peak of the responses in the oocyte illustrated in Fig 5.1 were 50 and 260 sec respectively. The mean values of the latency and the time-to-peak obtained from six mRNA-injected oocytes for the GnRH response were  $56 \pm 32$  sec and  $194 \pm 59$  sec, respectively(mean  $\pm$  SD). The maximum amplitude of the GnRH response (1  $\mu$ M) was 200 nA , this oocyte is shown in Fig. 5.1. The mean values of the maximum amplitude for membrane current induced by GnRH (1  $\mu$ M) were  $187 \pm 82$  nA (n=6).

#### Dose-Dependence of the GnRH Response

The size and frequency of the current fluctuations induced by GnRH were dependent on the applied concentration of the peptide as shown in Fig. 5.2,

Figure. 5.1. Membrane currents induced by the application of GnRH in an mRNA-injected *Xenopus* oocyte.

The oocyte was injected with approximately 50 ng of poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands and incubated for 6 days. Whole-cell current records were obtained using two-electrode voltage-clamp techniques. The holding potential was -60 mV. Current fluctuations superimposed on a sustained current evoked by the application of 1  $\mu$ M GnRH (gonadotropin-releasing hormone). The point of application of peptide is indicated by an arrow, washout with standard solution followed. GnRH was introduced to and removed from the experimental chamber by perfusion. Inward currents are shown as downward deflections in this and all other figures.

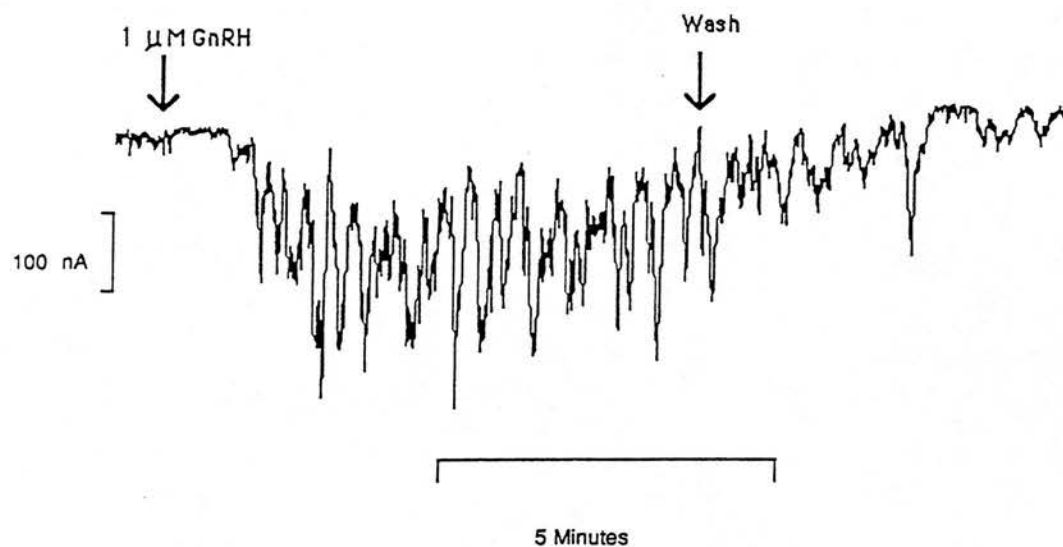
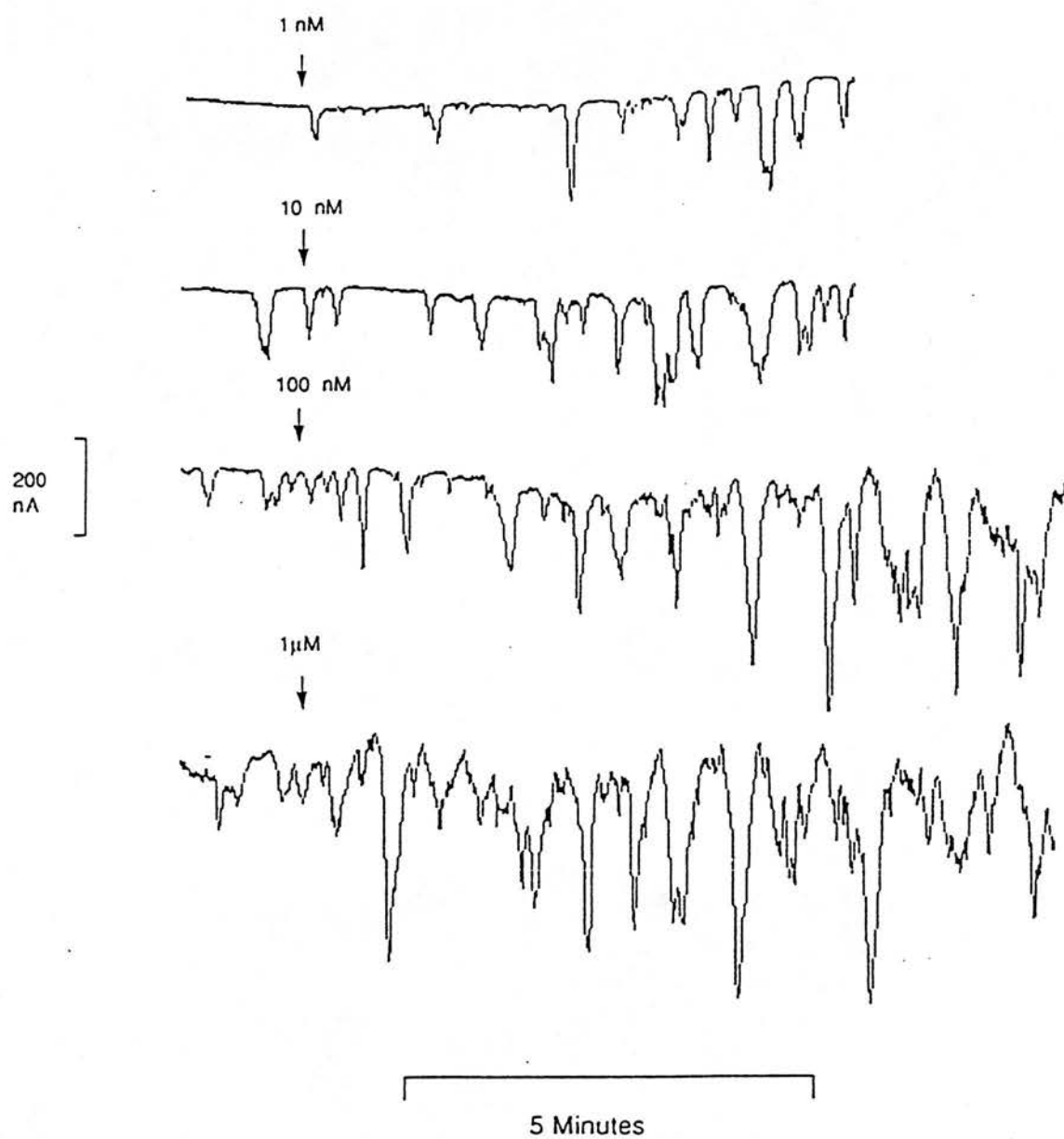


Figure. 5.2. Dose-dependence of the GnRH response.

Recordings made in *Xenopus* oocytes 4 days after injection of total poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands. The oocyte was clamped at -60 mV. Serial recordings were obtained by increasing the concentrations of GnRH in the bathing medium by perfusion in steps as indicated by arrows. Current and time calibrations are the same for all traces.



where all traces were obtained from the same oocyte, by increasing the peptide concentration. At the concentration of 1 nM GnRH, individual low frequency inward current fluctuations could be observed. As the concentration of GnRH was increased, the current fluctuations became larger and more frequent, resulting in a sustained inward current with superimposed current fluctuations. Random application of the different concentrations of GnRH were tried, and the dose-dependence of the response was assured qualitatively. An agonist of GnRH receptors (buserelin) was more potent in evoking current responses than GnRH at the same concentration (not illustrated). Selected oocytes (n=5) which showed only small responses to GnRH were tested with GnRH agonist (buserelin) at the same concentration (1  $\mu$ M). The maximum current amplitude was  $16 \pm 12$  nA for GnRH and  $228 \pm 101$  nA for GnRH agonist ( $P=0.025$ , n=5, Wilcoxon matched-pairs signed-rank test) indicating that GnRH agonist was much more potent than GnRH in inducing current responses.

### **Inhibition of the GnRH Response by $\text{Cl}^-$ Channel Blockers**

The current response evoked by GnRH was inhibited by the application of  $\text{Cl}^-$  channel blockers such as 9-anthracene carboxylic acid (9-AC), Diphenylamine-2-carboxylate (DPC), N-phenylanthranilic acid (NPAA) and 4,4'-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (see Wangemann, Wittner, Di Stefano *et al.* 1986 and Bretag, 1987 for reviews). An example of the blockade of the GnRH response by 9-AC, known to block  $\text{Cl}^-$  channels in renal epithelia (Oberleithner, Ritter, Lang & Guggino, 1983) and in *Xenopus* oocytes (Boton, Dascal, Gillo & Lass, 1989), is illustrated in Fig. 5.3. When GnRH (0.1  $\mu$ M) was applied to the oocyte a typical response was evoked, which was inhibited by the application of 9-AC (1 mM) in the continued presence of 0.1  $\mu$ M GnRH. When the inhibitor 9-AC was washed out with solution containing 0.1  $\mu$ M GnRH, the oscillating current response reappeared. Similarly, the GnRH response (1  $\mu$ M) was reversibly blocked by NPAA (0.1 mM), as shown in Figure 5.4. DPC, another  $\text{Cl}^-$  channel blocker described to be effective in epithelia (Di Stefano, Wittner, Schlatter *et al.*, 1985), partially inhibited the GnRH response at a concentration of 0.1  $\mu$ M, and a complete blockade was obtained with 0.1 mM. DIDS (a classical  $\text{Cl}^-$  channel blocker) abolished the GnRH response at the concentration of 0.1 mM (not shown). Current responses evoked by the GnRH super agonist buserelin were similarly inhibited by these  $\text{Cl}^-$  channel blockers. The results suggest that current responses induced by GnRH or GnRH agonist are dependent on the activity of the  $\text{Cl}^-$  channels.

Figure. 5.3. Inhibition of the GnRH response by the  $\text{Cl}^-$  channel blocker, 9-AC.

The current response was evoked by the application of  $0.1 \mu\text{M}$  GnRH and was reversibly blocked by  $1 \text{ mM}$  9-AC (9-anthracene carboxylic acid) in an mRNA-injected oocyte. The recovered GnRH response disappeared when GnRH was washed out. Arrows indicate the point of chemical application or washing-out with standard solution.



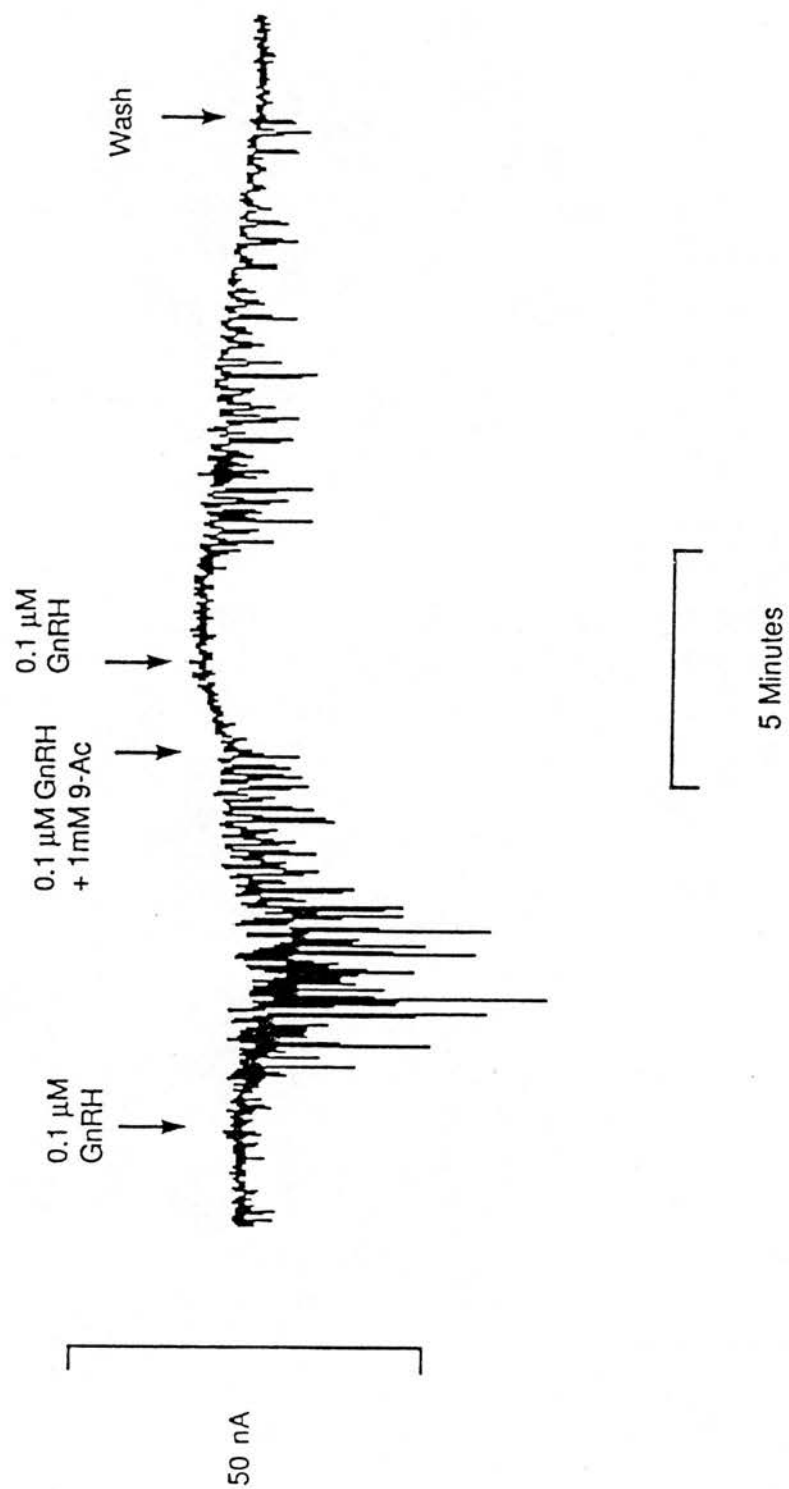
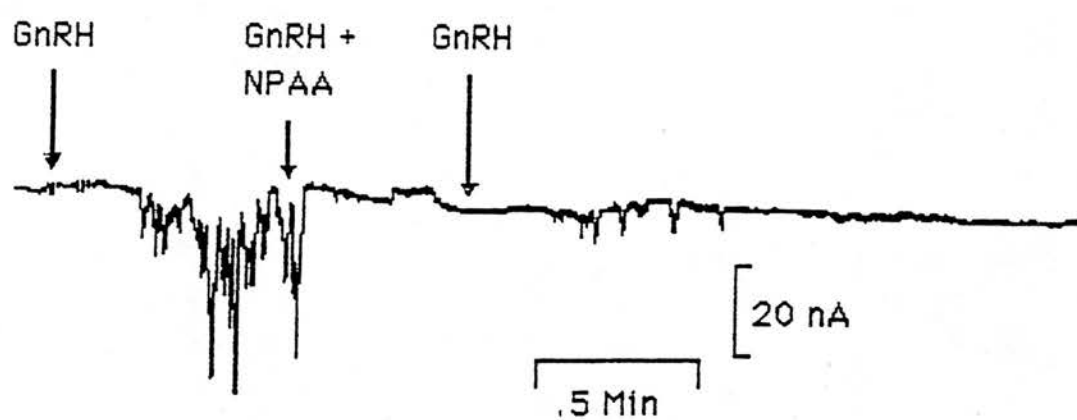


Figure. 5.4. Inhibition of the GnRH response by the  $\text{Cl}^-$  channel blocker, NPAA.

The current response was evoked by the application of  $1\ \mu\text{M}$  GnRH and was reversibly blocked by  $0.1\ \text{mM}$  NPAA ( N-phenylanthranilic acid) in an mRNA-injected oocyte. Arrows indicate the point of chemical application or washing-out .



### Reversal Potential of the GnRH Response

The type of ions responsible for the GnRH response can be identified by measuring the reversal potential of the response. Since currents evoked by GnRH fluctuate and vary with time, two different methods were used to estimate the reversal potential. Firstly, the reversal potential of the current fluctuations was estimated by changing the holding potential as described by Gillo, Lass, Nadler & Oron (1987) and by Boton, Dascal, Gillo & Lass (1989). The maximum amplitude of the current fluctuations was measured at different membrane potentials held for 1 - 1.5 min in oocytes which were continuously exposed to GnRH. An example of such an oocyte is shown in Figure 5.5. In this oocyte, the usual response to GnRH was initiated at a holding potential of -60mV. The holding potential was then changed in a step-wise manner, until at a holding potential of -25 mV the current flow was negligible. At holding potentials more positive than -25 mV the current reversed and became outward. The maximum current amplitude at each holding potential was plotted against the membrane potential (Fig. 5.6 open circles). During these measurements, the oocyte response to the peptide did not desensitize significantly. The I-V relationship was plotted and fitted using regression analysis, and the reversal potential was estimated from the point where the fitted line crossed the axis of the membrane potential  $V_m$  (the zero-current potential). The correlation coefficient of the fitted solid line shown in Fig. 5.6 was 0.987, and the reversal potential for the current was -26.3 mV. The mean estimated reversal potential ( $n=5$ ) for the current induced by buserelin or GnRH was  $-24.5 \pm 3.2$  mV. The correlation coefficient of fitted lines in these 5 examples was higher than 0.95, indicating the consistency of this method for estimating the reversal potential. It is to be noted that the I-V curve generally showed an outward rectification at negative membrane potentials (usually at potentials more negative than -50mV). The second method used for estimating the reversal potential was the ramp method (Boton, Dascal, Gillo & Lass, 1989; Gillo, Lass, Nadler & Oron, 1987; Dascal, Landau & Lass, 1984; Takahashi T, Neher E, Sakmann B 1987). 0.8 - 1.0 sec ramp-command voltages with slopes of 100 - 150 mV/sec were used in the present study. Similar reversal potentials of  $-25 \pm 3.2$  mV were obtained from this method ( $n=3$ ).

Dependence of the GnRH-induced current on  $Cl^-$  ions was further assured by changing the external concentration of  $Cl^-$ . The concentration of  $Cl^-$  was reduced to half (59.8 mM) by substituting the impermeant anion methanesulphonate for chloride. The linear portion of the I-V relationship in half  $Cl^-$  was plotted against

Figure. 5.5. Current-voltage (I-V) relation of the response induced by GnRH.

The application of GnRH ( $1\ \mu\text{M}$ ) produced a typical GnRH response in an oocyte clamped at  $-60\text{mV}$ , of inward current fluctuations. Changing the holding potential to the values indicated produced a change in the direction maximum amplitude of the GnRH-induced currents.

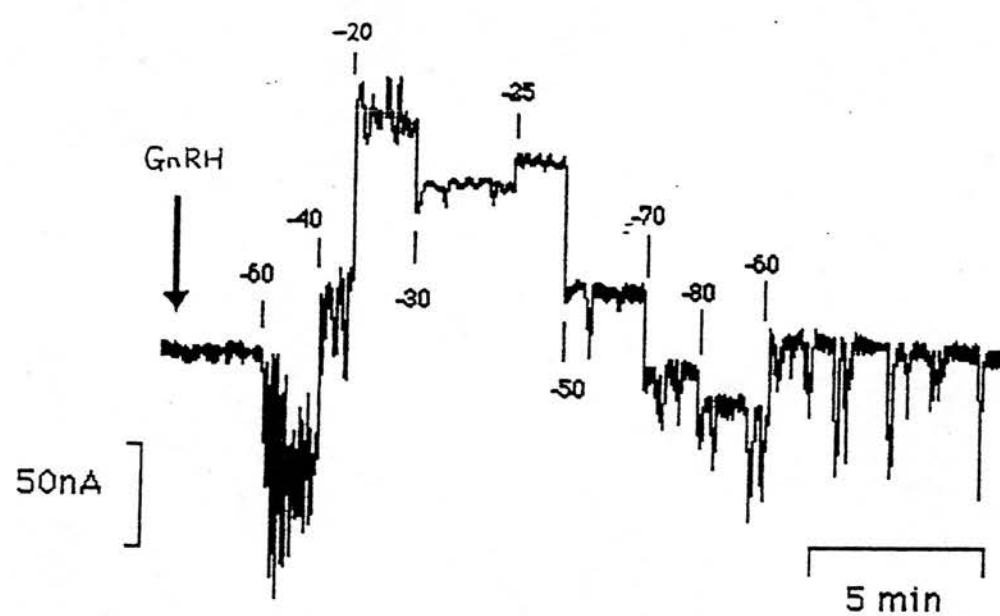
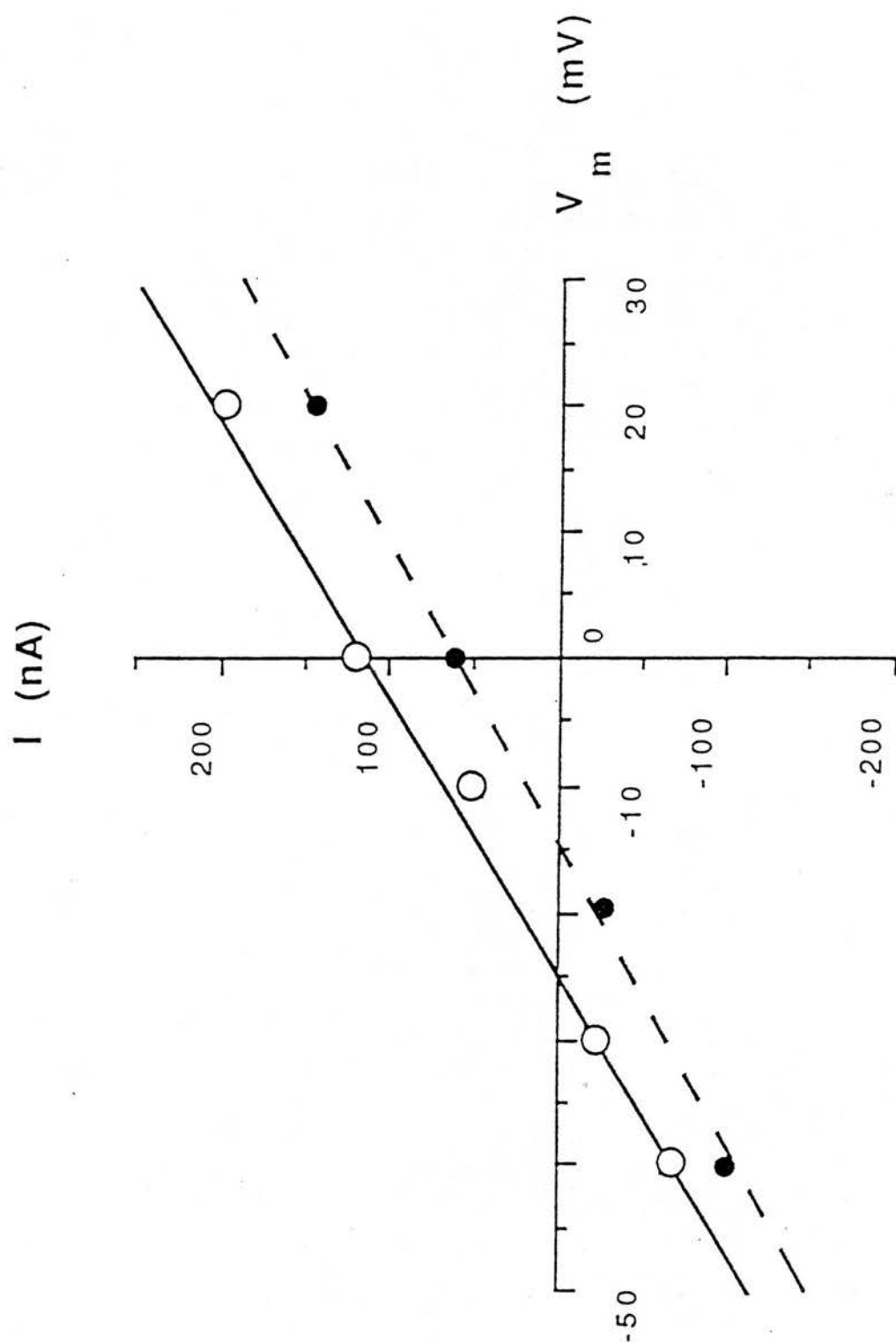


Figure. 5.6. Current-voltage (I-V) relation of the response induced by buserelin (an agonist of GnRH receptors).

The observed maximum amplitude of current fluctuations induced by buserelin were plotted against the holding potential. Open and filled circles, obtained from the same oocyte, were recorded in normal  $[\text{Cl}^-]_o$  (119.6 mM) and in half  $[\text{Cl}^-]_o$  (59.8 mM) solutions, respectively. The lines were fitted by linear regression analysis.





the holding potential (filled circles in Fig. 5.6) The fitted dashed line ( $r = 0.998$ ) showed a parallel shift and the calculated reversal potential was  $-14.4$  mV. The mean reversal potentials for currents induced by GnRH recorded from the same oocytes in normal and half  $\text{Cl}^-$  solutions were  $-25.2 \pm 3.0$  mV and  $-11.0 \pm 3.0$  mV ( $n=3$ ), respectively. Therefore the average depolarizing shift of the reversal potential by halving the concentration of  $\text{Cl}^-$  was  $14.2$  mV. This value is in a good agreement with the expected value of  $17.5$  mV given by the Nernst equation  $E_{\text{Cl}^-} = RT/F \log_{10} [\text{Cl}^-]_0 / [\text{Cl}^-]_o$  or  $E_{\text{Cl}^-} = 58.17 \log_{10} [\text{Cl}^-]_o / [\text{Cl}^-]_o$  at  $20^\circ\text{C}$ ;  $E_{\text{Cl}^-}$  is the reversal potential for  $\text{Cl}^-$  and the constants  $R$ ,  $T$  and  $F$  have conventional meanings. In addition, the reversal potential of the GnRH response was not significantly changed when the external concentration of  $\text{K}^+$  or  $\text{Na}^+$  were changed to  $10$  mM and  $57.5$  mM respectively ( $n=7$ ).

### Dependence of the GnRH Response on $\text{Ca}^{2+}$

Since other exogenous receptors expressed in *Xenopus* oocytes have been shown to produce current responses dependent on the activity of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels (see Dascal, 1987 and Snutch, 1988 for reviews), the dependence of the  $\text{Cl}^-$  channel activity on internal  $\text{Ca}^{2+}$  was examined for the GnRH response. The GnRH response was inhibited by TMB-8 (8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride), a blocker of intracellular  $\text{Ca}^{2+}$  release (Chiou & Malagodi, 1975) ( $n=4$ ). An example is shown in Fig. 5.7. TMB-8 was applied to an mRNA-injected oocyte at the concentration of  $0.2$  mM with  $1$   $\mu\text{M}$  GnRH; no significant response occurred in membrane current. TMB-8 was then removed from the bathing solution in the continued presence of GnRH, and the GnRH response was elicited. The response was suppressed when TMB-8 was reintroduced.

The calmodulin inhibitor pimozide has been shown to interfere with GnRH-stimulated LH (luteinizing hormone) release from pituitary cell cultures (Conn, Rodgers & Sheffield). Therefore the calmodulin inhibitor chlordiazepoxide was also tested on mRNA-injected oocytes. The GnRH response was inhibited by the addition of  $0.2 - 1$   $\mu\text{M}$  chlordiazepoxide ( $n=4$ ). An example of the inhibition induced by chlordiazepoxide is shown in Figure 5.8. In this oocyte,  $1$   $\mu\text{M}$  GnRH elicited the usual response. The further addition of  $1$   $\mu\text{M}$  chlordiazepoxide inhibited the response. Upon washing the oocyte with  $1\text{M}$  GnRH the response was partially recovered, despite some deterioration in the cell condition.

Figure. 5.7 Suppression of the GnRH response by a blocker of intracellular  $\text{Ca}^{2+}$  release, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8).

GnRH was firstly applied to an oocyte with TMB-8 (2 mM). The GnRH response was elicited when TMB-8 was removed from the bathing solution and inhibited again when it was reintroduced. The points of chemical applications are indicated by arrows.

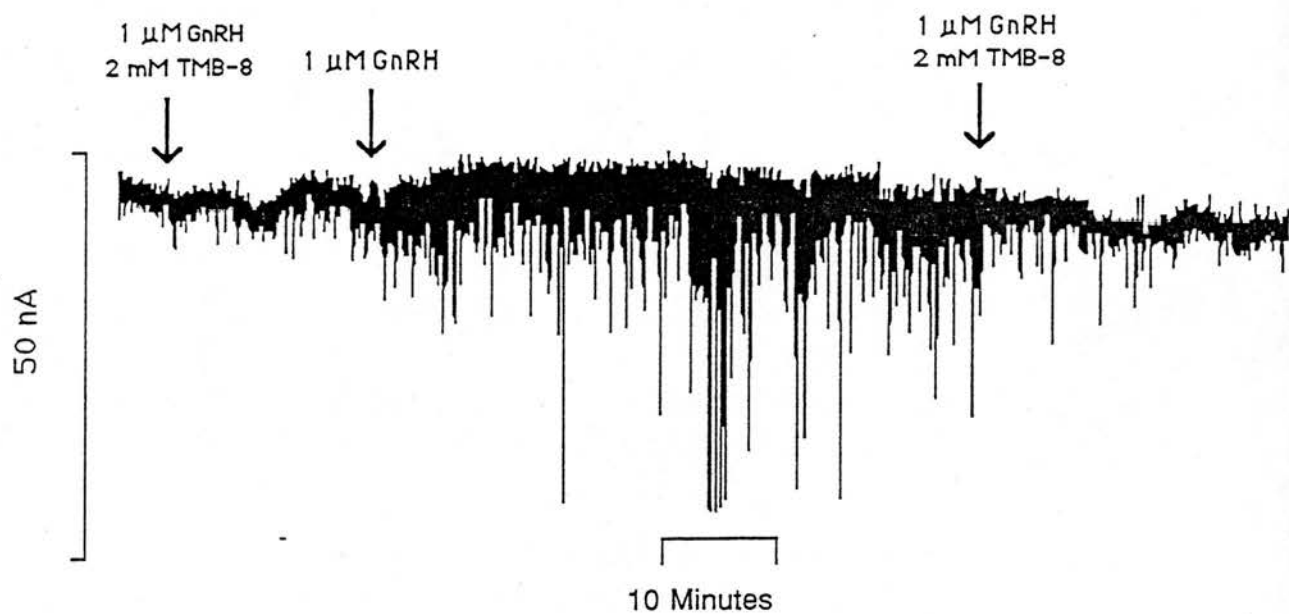
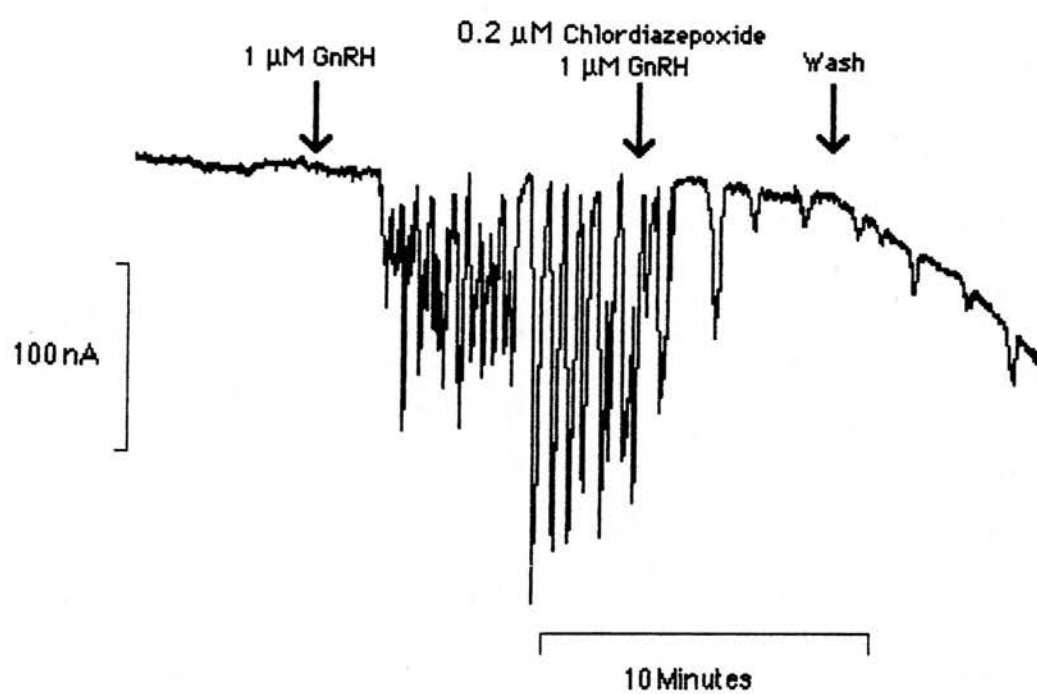


Figure. 5.8 Suppression of the GnRH response by a calmodulin inhibitor, chlordiazepoxide

The addition of GnRH ( $1\ \mu\text{M}$ ) induced inward current fluctuations which were inhibited upon the addition of chlordiazepoxide ( $0.2\ \mu\text{M}$ ). Upon washing the oocyte with GnRH ( $1\ \mu\text{M}$ ) the GnRH response began to recover.



## **Inhibition of the GnRH response by pertussis toxin**

Assuming that the GnRH receptor would be linked to a second messenger system which releases intracellular calcium, it is probable that this involves the activation of a G-protein linked to phospholipase-C. It has been established that pertussis toxin selectively catalyses the ADP ribosylation of the  $\alpha$  subunits of the G-proteins,  $G_o$  and  $G_i$  (Gilman, 1987) and has been used to provide an insight into the functions of these G-proteins. Therefore, the effect of pertussis toxin was studied in mRNA-injected oocytes. Pre-incubation with pertussis toxin ( $2\mu\text{g/ml}$ ) for 18hr completely abolished the response to  $1\mu\text{M}$  GnRH ( $n=3$ ). An example of the effect of treatment with pertussis toxin on the GnRH response is shown in Figure 5.9 A. In this oocyte the application of GnRH ( $1\mu\text{M}$ ) produced no response. In contrast, oocytes from the same donor, which had not been treated with pertussis toxin ( $n=3$ ) were responsive to GnRH. An example of such a control oocyte is shown in Figure 5.9 B, where the application of GnRH ( $1\mu\text{M}$ ) produces the usual inward current fluctuations.

## **Attempted cloning of the GnRH receptor**

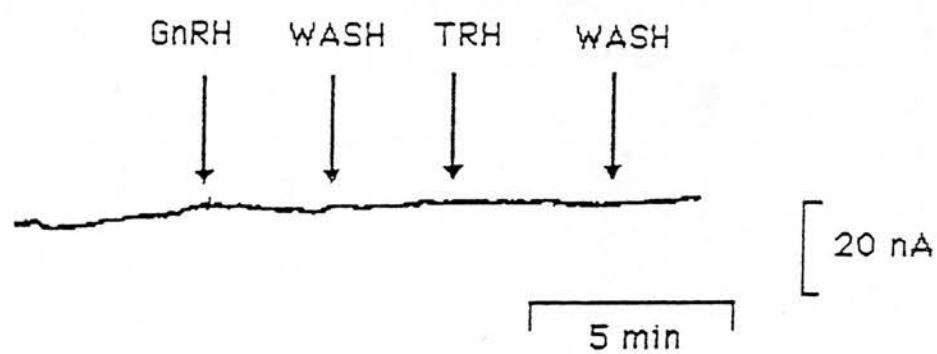
The poly(A)+ mRNA from the rat anterior pituitary gland will contain mRNA of various sizes which encode the various pituitary components. In order to isolate the clone which encodes the GnRH receptor a complementary DNA library must be made from the poly(A)+ mRNA and this library must then be screened to isolate the GnRH receptor clone. Screening can be done using the *Xenopus* oocyte expression system, by injecting successively smaller fractions of the library and following the expressed activity until a single clone is isolated which codes for the GnRH receptor. Such a strategy has been successfully used to clone the serotonin (Julius, McDermott, Axel *et al*, 1983), substance K (Masu, Nakayama, Tamaki *et al* 1988). This screening process is greatly facilitated by having a smaller cDNA library and therefore the initial step in such a study is to isolate a fraction of the poly (A)+ mRNA which gives rise to GnRH activity according to size.

### **Fractionation of the mRNA**

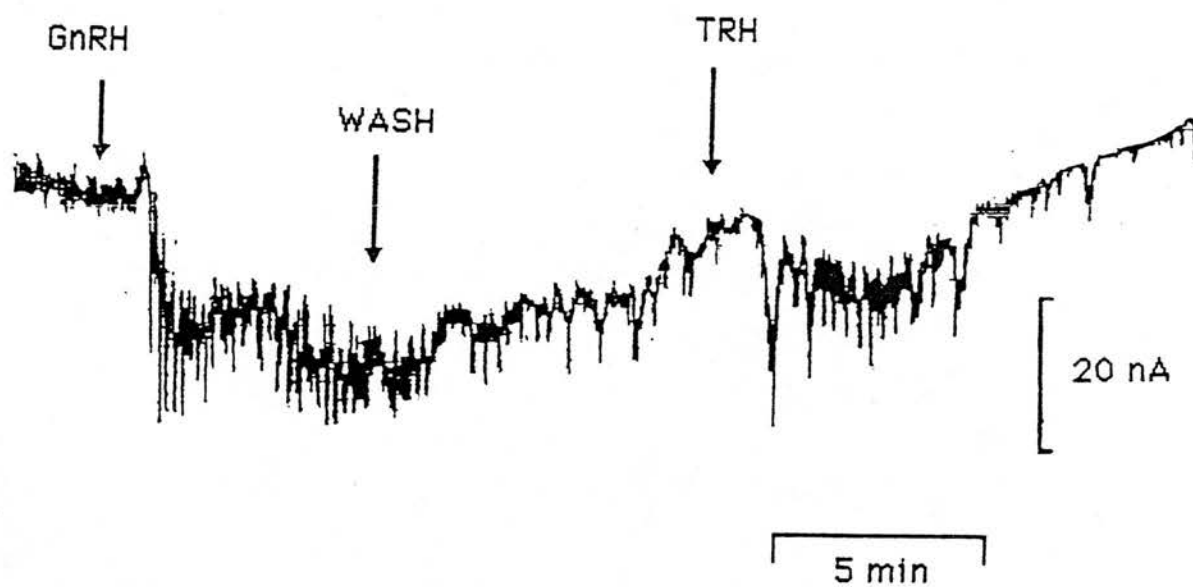
To determine the size of the RNA species coding for the GnRH receptor, size fractionation of total RNA derived from rat anterior pituitary glands was carried out on NaDodSO<sub>4</sub> sucrose-density gradients (Richter, Schmale, Ivell & Schmidt, 1980). These size-selected fractions were injected separately into oocytes.

Figure 5.9. The effect of pertussis toxin on the GnRH response induced by injection of the *Xenopus* oocytes with rat pituitary poly (A)+ mRNA. (A) shows the effect of preincubation of an oocyte with pertussis toxin (2 $\mu$ g/ml) for 18 hr. The application of GnRH (1 $\mu$ M) and TRH (1 $\mu$ M) are indicated by the arrows. An oocyte from the same donor, also injected with the mRNA, but not treated with pertussis toxin is shown in B. The addition of GnRH and TRH (both at 1 $\mu$ M) elicit inward current fluctuations.

A



B





Current responses were evoked by the application of GnRH to oocytes injected with fractions eluting between the 18S and 28S peaks. An example of such an oocyte (injected with fractions 9-11) is shown in figure 5.10, where the application of TRH (1 $\mu$ M) produces no response whilst the application of GnRH (1 $\mu$ M) produces current fluctuations. The maximum response was obtained from oocytes injected with fraction 11, with a current response as large as 320 nA in amplitude recorded. The size-fractionation of RNA on a sucrose density gradient is shown in Fig. 5. 11. No responses were obtained from RNA fractions smaller than 18S or larger than 28S indicating that the mRNA's encoding for the GnRH receptor are between 3 and 4,000 base-pairs (bp) in size.

#### Attempted cloning of the GnRH receptor

A rat pituitary, size-selected cDNA library was constructed in the plasmid vector Lambda Zap II, by Dr K.A.Eidne and co-workers, containing inserts of cDNA of greater than 2,000 bp. This pituitary cDNA library contained about ~10,000,000 individual clones. To screen this library the functional expression assay in *Xenopus* oocytes was used. The library was split into 25 pools of 40,000 clones. Messenger RNA was transcribed from these pools and injected into oocytes. After an incubation period of two to four days, these oocytes were examined electrophysiologically for a response to GnRH. Initial studies showed responses from certain fractions of the cDNA library, but the responses were not reproducible. Further work is needed to isolate the GnRH clone.

## DISCUSSION

The results presented above indicate that the responses to GnRH or the GnRH super agonist (buserelin) measured in *Xenopus* oocytes injected with rat pituitary mRNA were mainly dependent on the activity of Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels. The GnRH response was inhibited by the Cl<sup>-</sup> channel blockers 9-AC, DPC, NPAA and DIDS. The oscillatory nature of the GnRH response resembles the current fluctuations induced by GnRH in rat gonadotropes (Croxtan, Ben-Jonathon & Armstrong, 1988). Increasing the GnRH concentrations from 1nM to 1 $\mu$ M increased the amplitude of these current fluctuations in a dose-dependent manner. Isolated rat pituitary glands *in vitro* doses of GnRH in the range 10<sup>-10</sup> to 10<sup>-7</sup> M gave rise to release of LH (Liu & Jackson, 1979), so the doses used in this study are comparable with those used in other experimental situations. The reversal potential for the GnRH response was approximately -25 mV, and this value agrees well with the reported reversal potential for Cl<sup>-</sup> in *Xenopus* oocytes (Dascal, Landau & Lass, 1984; Kusano, Miledi & Stinnakre, 1982; Busa &

Figure 5.10 Shows the response to GnRH (1uM) from an oocyte injected with fraction 11 of poly (A)+ mRNA obtained from a sucrose-density gradient. There was no response to TRH (1uM) from this oocyte.

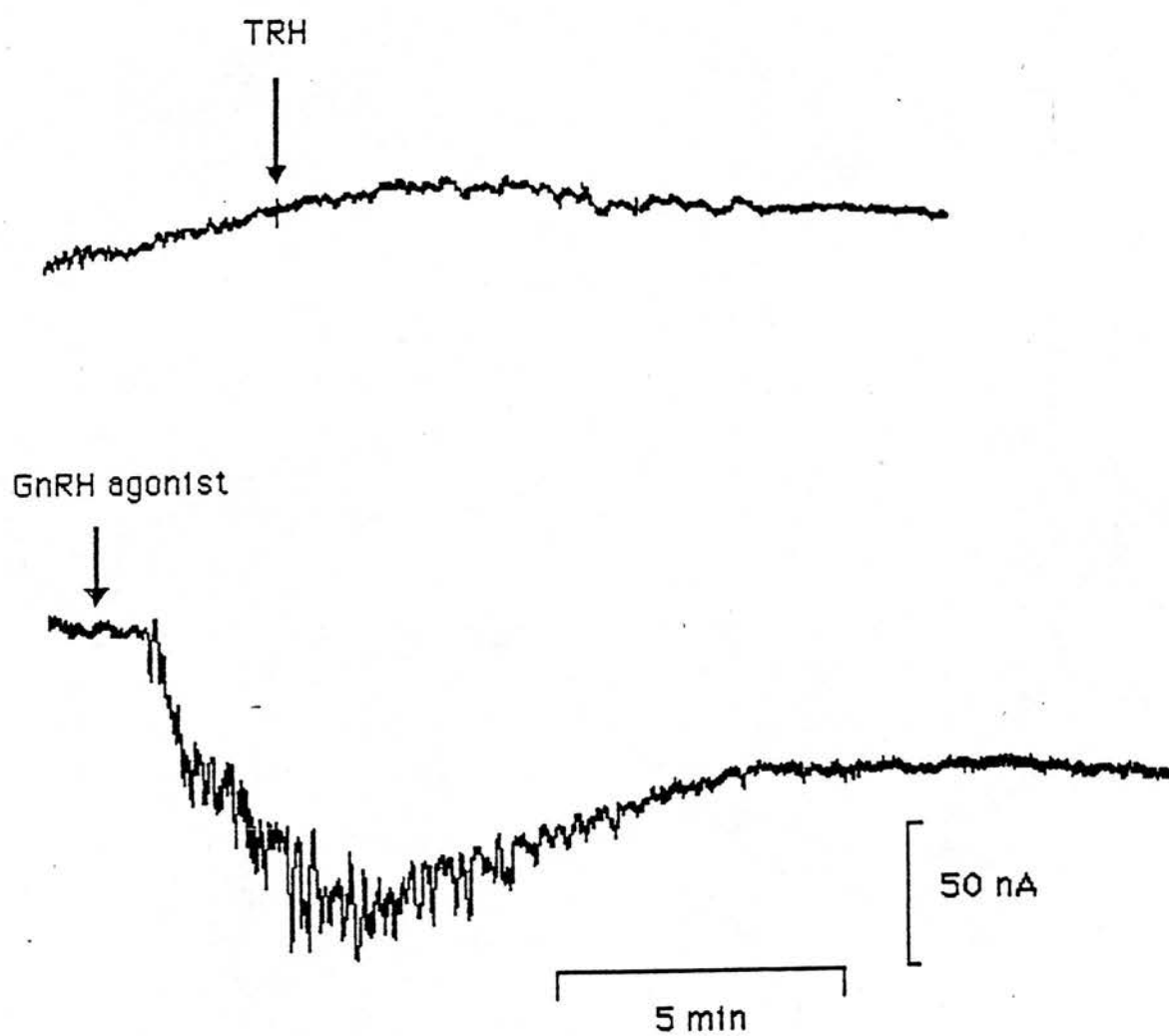
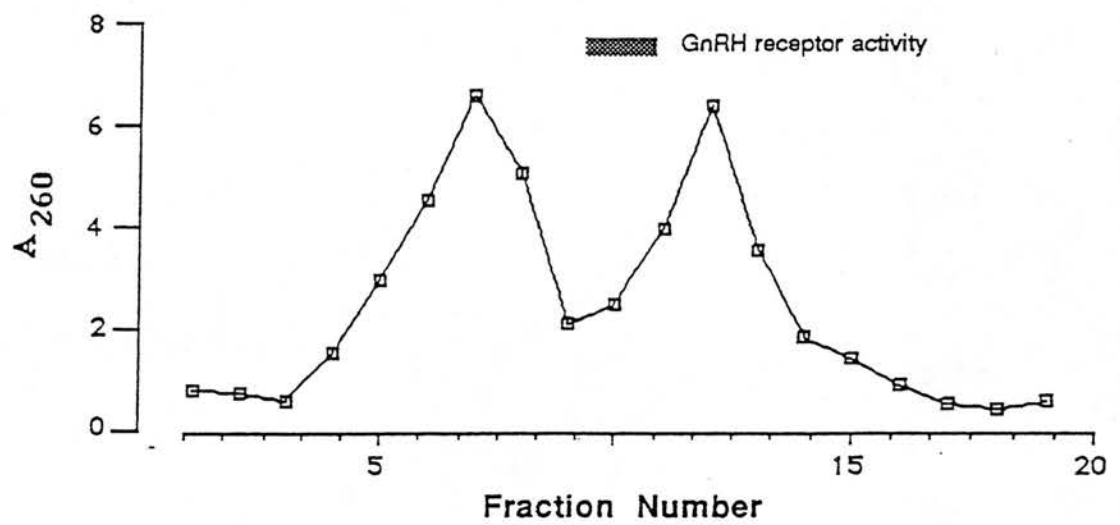


Figure. 5.11. Sucrose-density gradient of poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands. Individual fractions were tested by injection of 50 nl into *Xenopus* oocytes for electrophysiological assay. The mRNA-injected oocytes were voltage clamped at -60 mV and exposed to 1  $\mu$ M of either GnRH or GnRH agonist. Horizontal bar indicates the position of the mRNA fraction (fractions 9 - 12) which gave rise to positive responses to the peptides.

### Gradient Fractionation of Rat Pituitary RNA



Nuccitelli, 1985). The outward rectification of the I-V curve at potentials more negative than -50mV found in this study have been reported to be characteristic of the Cl<sup>-</sup> current in *Xenopus* oocytes (Miledi & Parker, 1984; Takahashi, Neher & Sakmann, 1987; Oosawa & Yamashita, 1989). This rectification has been ascribed to changes in the gating and conductance of single Cl<sup>-</sup> channels (Levitan, 1988).

Further evidence for the dependency of the GnRH response on Cl<sup>-</sup> was provided by the observed shift in the reversal potential with the change in concentration of external Cl<sup>-</sup>. The depolarizing shift of the reversal potential produced by halving the external Cl<sup>-</sup> concentration was 14.2 mV, similar to the value of 17.5 mV predicted by the Nernst equation for a Cl<sup>-</sup> electrode. Also, the reversal potential of the GnRH response was not significantly changed when the external concentration of K<sup>+</sup> or Na<sup>+</sup> were changed

In the pituitary gonadotrope, the application of GnRH (10nM) does not activate a Cl<sup>-</sup> current, but a K<sup>+</sup> current dependent on internal Ca<sup>2+</sup> (Mason & Waring, 1986; Croxton, Ben-Jonathon & Armstrong, 1988). In the present study, the activity of the Cl<sup>-</sup> channel responsible for the GnRH response was also dependent on internal Ca<sup>2+</sup>. TMB-8, a blocker of intracellular Ca<sup>2+</sup> release (Chiou & Malagodi, 1975), inhibited the GnRH response. A rise in the intracellular concentration of Ca<sup>2+</sup> would appear to be important in the response to GnRH in *Xenopus* oocytes. GnRH has also been demonstrated to increase the intracellular concentration of Ca<sup>2+</sup> in gonadotropes (Clapper & Conn, 1985). In the *Xenopus* oocyte TMB-8 completely inhibited the response to GnRH which would suggest that the response is due, entirely, to the release of calcium from intracellular stores, and that calcium entry from the extracellular medium played little part in the response. Indeed it has been shown that extracellular calcium is unimportant in the response to other expressed receptors in *Xenopus* oocytes. However, in gonadotropes, it has been observed that GnRH-stimulated release of LH involves both extracellular Ca-dependent and -independent mechanisms (Chang, Stojilkovic, Graeter & Catt, 1988; Smith, Wakefield, King *et al.*, 1987; Hansen, McArdle & Conn, 1987). In single gonadotropes, the result of the addition of GnRH is to produce oscillations in the internal concentration of calcium, as measured with the calcium-sensitive fluorescent dye, Fura-2 (Shangold, Murphy and Millar, 1988). Such oscillations in intracellular calcium have been seen in a variety of cells and notably in mouse (Cuthbertson & Cobbold, 1985) and hamster (Miyazaki, 1988) oocytes. Such oscillations vary in frequency with the concentration of the applied agonist, and the external

concentration of calcium. Hence, the *Xenopus* oocyte is a good system for the study of the GnRH receptor and its second messenger systems, but there are some limitations because there are the endogenous factors to consider and thus, a direct comparison with the response in the gonadotrope is difficult.

Inhibiting calmodulin with pimozide has been reported to inhibit GnRH-stimulated LH (luteinizing hormone) release by binding to calmodulin in rat pituitary cell cultures (Conn, Rogers & Sheffield, 1981). This finding supports the role of calcium mobilisation and calmodulin activation in the mediation of GnRH-stimulated LH release. The present data show that the GnRH response in mRNA-injected *Xenopus* oocytes was also inhibited by the calmodulin inhibitor chlordiazepoxide suggesting that these responses may be dependent on calmodulin which controls intracellular  $\text{Ca}^{2+}$ . It is therefore likely that the GnRH response is dependent on the mobilization of intracellular  $\text{Ca}^{2+}$  for the activation of  $\text{Cl}^-$  channels.

The direction of the current carried by  $\text{Cl}^-$  ( $I_{\text{Cl}^-}$ ) in response to GnRH or busserelin can be described by the equation  $I_{\text{Cl}^-} = g_{\text{Cl}^-} (E - E_{\text{Cl}^-})$  where  $E$  and  $E_{\text{Cl}^-}$  indicate the membrane potential and the reversal potential for  $\text{Cl}^-$ , respectively. Under voltage-clamp conditions when the holding potential is set at -60 mV (close to the resting potential), the driving force for  $\text{Cl}^-$ , i.e.  $(E - E_{\text{Cl}^-})$  is negative because the equilibrium potential for  $\text{Cl}^-$  is approximately -25 mV, since the  $\text{Cl}^-$  conductance ( $g_{\text{Cl}^-}$ ) is positive  $I_{\text{Cl}^-}$  must therefore be negative. Negative (or inward) current means an efflux of  $\text{Cl}^-$ . This increase in chloride conductance would explain the depolarizing membrane-potential change observed with intracellular recording techniques in Section 3. Under current clamp conditions, the resting potential of oocytes (approximately -60 mV) is more negative than the equilibrium potential for  $\text{Cl}^-$  (approximately -25 mV) and hence the oocyte membrane potential shifts toward this equilibrium potential, resulting in a membrane depolarization as the membrane becomes permeable to  $\text{Cl}^-$  in response to GnRH or busserelin.

So far, exogenous receptors expressed in *Xenopus* oocytes for many receptors, including those for acetylcholine, serotonin, TRH and substance K, produce current responses dependent on the activity of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (see Dascal, 1987 and Snutch, 1988 for review). Recently, the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance in *Xenopus* oocytes was further classified into two components, a fast, transient, and a slow component (Boton, Dascal, Gillo & Lass, 1989). These two components have different  $\text{Ca}^{2+}$  dependence of activation and inactivation and different sensitivity to a  $\text{Cl}^-$  channel blocker. The

slow component was less sensitive to 9-AC (the amplitude of slow current was only reduced by 59 % by 2 mM 9-AC). The  $\text{Cl}^-$  conductance in response to GnRH in the present study would appear to resemble the slow component of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance in its time course but is more sensitive to 9-AC. We have noted that higher concentrations of 9-AC and DPC, potent  $\text{Cl}^-$  channel blockers in epithelia (Oberleithner, Ritter, Lang & Guggino, 1983; Di Stefano, Witter, Schlatter *et al*, 1985), were required for an effect in *Xenopus* oocytes. It is possible that ion channels in undifferentiated cells such as *Xenopus* oocytes are less sensitive to these blockers than those in epithelia. Indeed, it has been shown that  $\text{Ca}^{2+}$  channels in mouse oocytes are less sensitive to a  $\text{Ca}^{2+}$  channel blocker, diltiazem, than in differentiated cells (Yoshida, 1986). In a recent paper on the expression of GnRH receptors from a pituitary cell line expressing GnRH receptors that a fast transient component to the application of GnRH was observed (Sealfon, Gillo, Mundamatton *et al* 1990). In the present study a fast component was not obtained. This difference in the response profile is, perhaps due to the different source or mRNA producing a higher density of GnRH receptors.

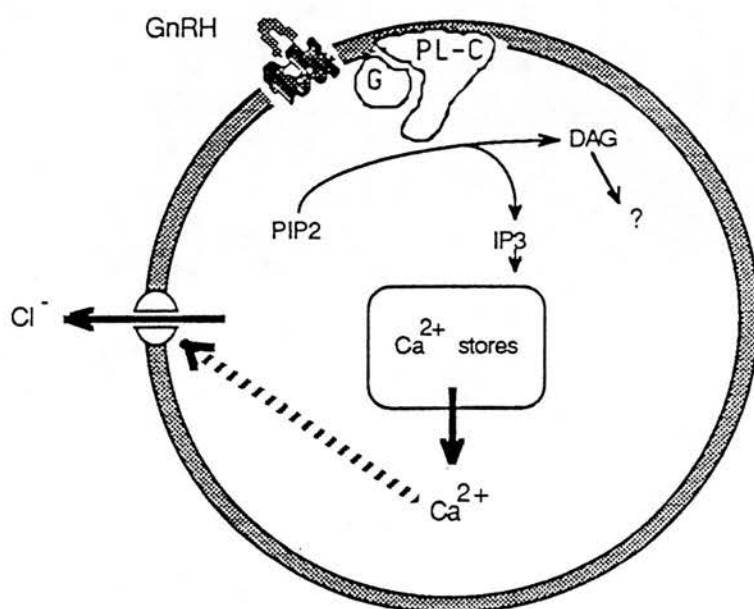
In the pituitary, gonadotropes have also been shown to have a G-protein which is activated by GnRH and is linked to the enzyme phospholipase-C which stimulates the breakdown of  $\text{PIP}_2$  (Andrews, Stanley, Huckle & Conn, 1986; Andrews & Conn, 1986). Reports have indicated that there are two classes of G-protein involved in receptor-phospholipase-C coupling, which are either sensitive or insensitive to pertussis toxin in several cell lines (Volpe, Naccache, Shefcyk *et al.*, 1985; Kikuchi, Kozawa, Kaibuchi *et al*, 1986; Masters, Martin, Harden & Brown, 1985 ; Hepler and Harden ,1986). On the basis of this study with pertussis toxin the GnRH receptor is linked to a G-protein which is pertussis toxin-sensitive. The *Xenopus* oocyte has also been shown to contain endogenous G-proteins (Sadler, Maller & Cooper, 1984; Goodhardt, Ferry, Buscaglia *et al.*, 1984). The question which arises is whether the response seen in the *Xenopus* oocyte is due to the activation of an endogenous G-protein, or whether the G-protein is that found in the rat pituitary and is expressed along with the GnRH receptor. As mentioned in the results section, a particular fraction of mRNA encodes the GnRH receptor. As G-proteins are known to be smaller than G-protein-linked receptors, it seems unlikely that a fraction of mRNA encoding the GnRH receptor would also encode its G-protein. Therefore the G-protein used by the GnRH receptor would appear to be endogenous to the *Xenopus* oocyte.



The GnRH receptor appears to stimulate the activation of phospholipase C, via a G-protein to produce a rise in intracellular calcium, the effect of which, as is well established in the *Xenopus* oocyte, is to cause opening of Cl<sup>-</sup> channels. The activation of phospholipase-C is also known to cause the production of diacylglycerol (DAG) (Berridge and Irvine, 1984). In gonadotropes, GnRH, in addition to causing a rise in Ca<sup>2+</sup>, produces a rise in DAG (Andrews & Conn, 1986). DAG is known to activate protein kinase C (PKC) (Nishizuka, 1984). This activation of PKC appears to mediate the GnRH action in the gonadotrope since the application of DAG provokes the release of LH (Conn, Ganong, Ebling *et al.*, 1985). It is interesting to note that if a continuous infusion of GnRH is given, the pituitary rapidly becomes insensitive to further stimulation (desensitisation) (Belchez, Plant, Nakai *et al.* 1978) and at least part of the desensitisation of the response to GnRH seen in gonadotropes is secondary to PKC depletion, as a similar desensitisation can be induced by activating PKC with phorbol esters (Clayton, 1988). Similarly in *Xenopus* oocytes the activation of PKC appears to cause desensitization as in a study of the expressed serotonin and acetylcholine receptor responses, the activation of PKC caused a reduction in the response to these two agonists (Moran & Dascal, 1989). A cell model depicting the events which occur in an oocyte expressing GnRH receptors, upon the exposure to GnRH, is shown in Figure 5.12. Receptor binding causes a membrane G-protein (G) to bind GTP and subsequently dissociate. The free  $\alpha$ -GTP then interacts with a phosphoinositide enzyme, phospholipase C (PLC) causing it to cleave membrane phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to yield inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> acts as a second messenger to release calcium from intracellular stores and the resulting rise in intracellular Ca<sup>2+</sup> in turn activates Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels present in the plasma membrane (Miledi, 1982; Miledi & Parker, 1984) to give the final current response.

The present study has shown that the rat anterior pituitary gland mRNA encoding the GnRH receptor can be translated in *Xenopus* oocytes. These receptors are able to couple up to G protein-linked second messenger systems present in *Xenopus* oocytes. The mechanism of this response would appear to depend on the activity of chloride channels which are endogenous in the oocytes, as has been found in studies of the expression of other receptors (Dascal, 1987; Snutch, 1988). The cloning of the GnRH receptor has, as yet proved unsuccessful. At the time of this study, there was uncertainty as to the quality of the oocytes. A seasonal problem has been encountered, lasting from January to April each year

**Figure 5.12** A cell model of the events which occur between the binding of GnRH to its receptor, and the activation of a  $\text{Cl}^-$  current in the *Xenopus* oocyte.



- G - G-protein
- PLC - phospholipase C
- PIP2 - phosphoinositol bisphosphate
- IP3 - inositol trisphosphate
- DAG - diacylglycerol

in which the animals imported from South Africa, are immature and this may have contributed to the lack of success in this part of the project. A further possibility is that the mRNA made from the cDNA library is unstable in the *Xenopus* oocyte unless a poly adenylated tail of sufficient length is added (Bernstein & Ross, 1989). A third possibility is that the cDNA library does not contain a sufficient GnRH receptor clones to allow such a procedure to be successful. The cloning of the GnRH receptor remains one of the primary objectives of the MRC Centre for Reproductive Biology in Edinburgh, under the supervision of Dr K.A. Eidne. Further progress in the area of GnRH agonists, which are widely used in clinically in the treatment of breast cancer, endometriosis and prostatic cancer etc (Frazer & Baird, 1987) would be greatly assisted by a knowledge of the molecular structure and function of the GnRH receptor.

## SECTION 6

### A Study of the response elicited by TRH receptors expressed in *Xenopus* oocytes using two-electrode voltage-clamp.

The results presented in this section form an investigation of the TRH response translated into *Xenopus* oocytes by the injection of rat anterior pituitary poly(A)<sup>+</sup> mRNA encoding TRH receptors

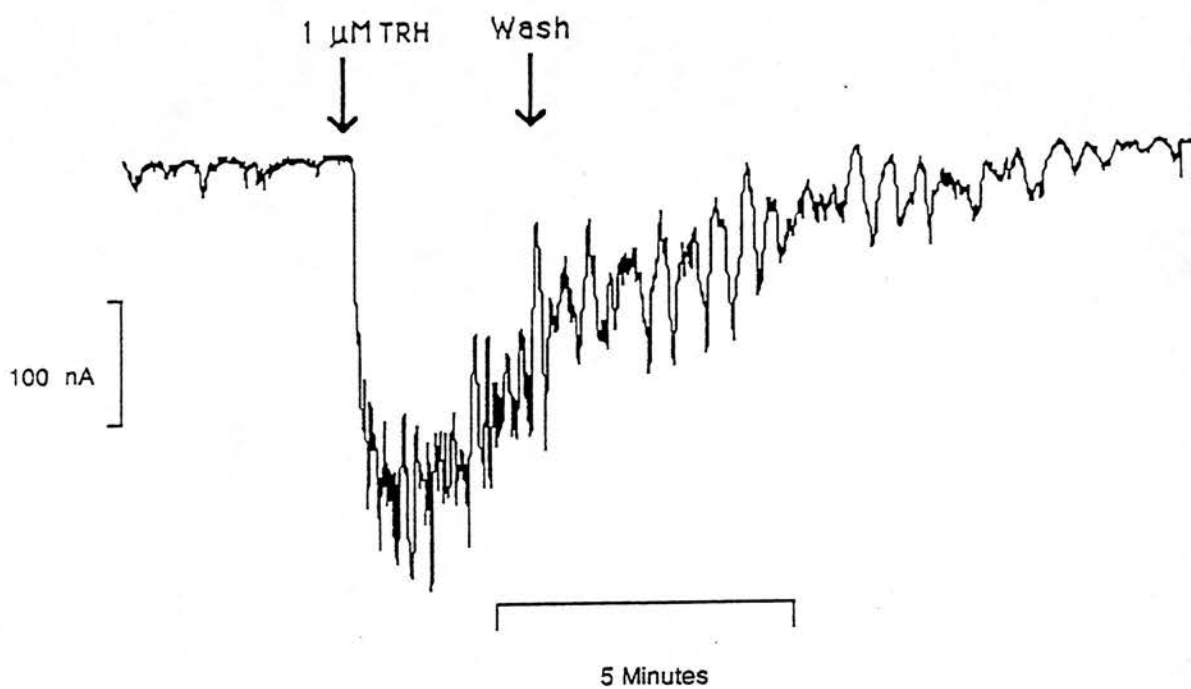
#### Current Responses induced by TRH

Membrane responses were observed in oocytes injected with poly(A)<sup>+</sup>-mRNA isolated from rat anterior pituitary glands following the application of TRH. Perfusion of TRH (1  $\mu$ M) into the external solution induced a slow, long-lasting inward current with superimposed fluctuations appearing with a latency of approximately 15 sec, reaching its peak about 95 sec after the application of peptide (Fig.6.1). As with the response to GnRH, as shown in Section 5, the current fluctuations became less frequent and smaller in size on washing out the peptide. Some oocytes responded to both GnRH and TRH. In this study of 50 successfully expressing oocytes, the expression of TRH receptors was more likely than that of GnRH receptors. 36% of oocytes were responsive to TRH only, and 12 (48%) responded both to GnRH and TRH. Whereas, a response to GnRH only was elicited in 24% of oocytes. There were also characteristic differences between the GnRH and TRH responses obtained from the same oocytes. Both the latency and the time-to-peak of the GnRH response were always longer than those of the TRH response. The latency and the time-to-peak of the responses to TRH in the oocyte illustrated in Fig.6.1 were 15 and 95 sec. The mean values of the latency and the time-to-peak obtained from six mRNA-injected oocytes were  $56 \pm 32$  sec (mean  $\pm$  SD) and  $194 \pm 59$  sec respectively for the GnRH response and  $26 \pm 19$  sec and  $92 \pm 43$  sec for the TRH. TRH responses exhibited significantly smaller values for both latency and time-to-peak than GnRH responses in each pair of data ( $P=0.025$ ,  $n=6$ , Wilcoxon matched-pairs signed-rank test). The maximum amplitude of the current was 410 nA for the TRH response (at 1  $\mu$ M) in the oocyte shown in Fig. 6.1. The mean values of the maximum amplitude for membrane current induced by TRH (1  $\mu$ M) were  $272 \pm 156$  nA ( $n=6$ ), larger than those elicited by 1  $\mu$ M GnRH, at  $187 \pm 82$  nA ( $n=6$ ).

To avoid desensitisation, the oocytes were usually exposed to peptide for short periods before being washed out. In oocytes continuously exposed to TRH

Figure. 6.1. Membrane currents induced by the application of TRH in an mRNA-injected *Xenopus* oocyte.

The oocyte was injected with approximately 50 ng of poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands and incubated for 6 days. Whole-cell current records were obtained using two-electrode voltage-clamp techniques. At the holding potential of -60 mV, current fluctuations superimposed on a transient current were evoked by the application of 1  $\mu$ M TRH (thyrotropin-releasing hormone). The point of application of TRH is indicated by an arrow, washout with standard solution followed. TRH was introduced to and removed from the experimental chamber by perfusion. Inward currents are shown as downward deflections in this and all subsequent figures.



however, as with GnRH, the response lasted for longer periods (longer than 20 min), but did eventually decay (desensitize). Oocytes also became refractory to repeated applications of TRH, as each successive current response showed longer latencies and smaller peak amplitudes ( $n=5$ ).

Control (uninjected or water-injected) *Xenopus* oocytes showed no changes in membrane current when exposed to TRH at concentrations ranging from 1nM to 1 $\mu$ M (not illustrated).

### **Inhibition of the TRH Response by $\text{Cl}^-$ Channel Blockers**

The current response evoked by TRH was inhibited by the application of  $\text{Cl}^-$  channel blockers such as 9-AC and DIDS (see Wangemann, Wittner, Di Stefano *et al.* 1986 and Bretag, 1987 for reviews). An example of the blockade of the TRH response by 9-AC is illustrated in Fig. 6.2. When TRH (0.1  $\mu$ M) was applied to the oocyte a typical response was evoked, which was inhibited by the application of 9-AC (1 mM) in the continued presence of 0.1  $\mu$ M TRH, but a complete block was not attained. When the inhibitor 9-AC was washed out with solution containing 0.1  $\mu$ M TRH, the oscillating current response became larger. DIDS (a classical  $\text{Cl}^-$  channel blocker) abolished the TRH response at the concentration of 0.1 mM (not shown). The results suggest that current responses induced by TRH, like those induced with GnRH or GnRH agonist are dependent on the activity of  $\text{Cl}^-$  channels.

### **Reversal Potential of the TRH Response**

The type of ions responsible for the TRH response was identified by measuring the reversal potential of the response. The reversal potential of the current fluctuations was estimated by changing the holding potential as mentioned previously. The maximum amplitude of the current fluctuations was measured at different membrane potentials held for 1 - 1.5 min in oocytes which were continuously exposed to TRH. An example of such an oocyte is shown in Figure 6.3 A. In this oocyte, a response to TRH (1 $\mu$ M) was initiated at a holding potential of -60mV. The holding potential was then changed in a step-wise manner, until at a holding potential of -25 mV the current flow was minimal. At holding potentials more positive than -25 mV the current reversed and became outward. The maximum current amplitude at each holding potential was plotted against the membrane potential (Fig. 6.4 open circles). During these measurements, the oocyte response to the peptide did not greatly desensitize. The I-V relationship was plotted and fitted using regression analysis, and the reversal potential (- 22 mV) was estimated from the point



Figure. 6.2. Inhibition of the GnRH response by a  $\text{Cl}^-$  channel blocker.

The current response was evoked by the application of  $1\ \mu\text{M}$  TRH and was reversibly suppressed by  $1\ \text{mM}$  9-AC (9-anthracene carboxylic acid) in an mRNA-injected oocyte. Arrows indicate the point of chemical application or washing-out with standard solution.

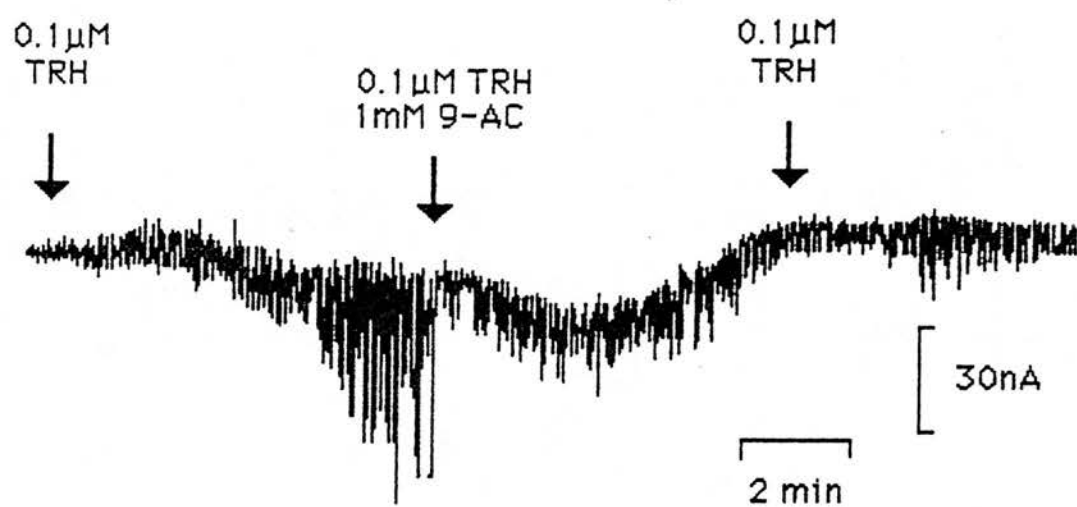
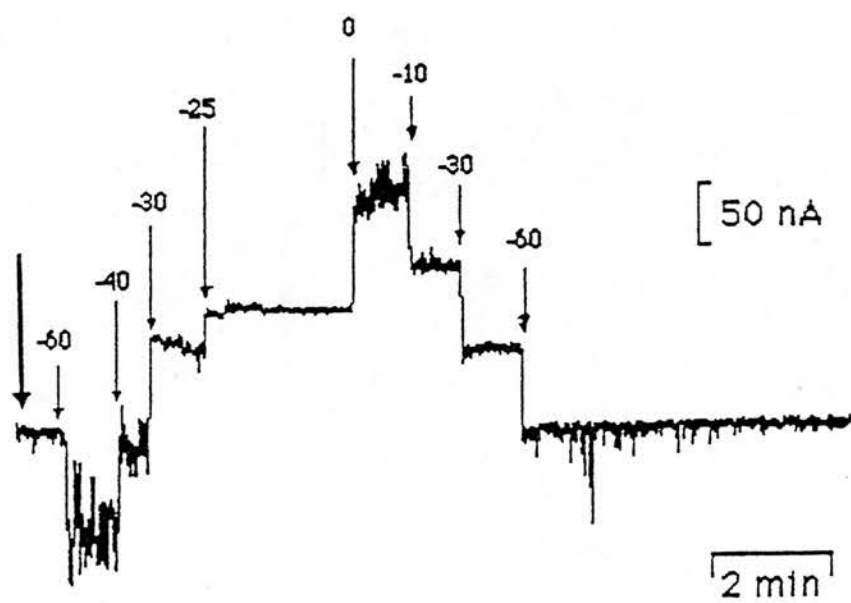


Figure. 6.3. Current-voltage (I-V) relation of the response induced by GnRH.

In A, the application of TRH (1  $\mu$ M) bathed in normal  $[\text{Cl}^-]_o$  (119.6 mM) produced a typical TRH response in an oocyte clamped at -60mV, of inward current fluctuations. Changing the holding potential to the values indicated by the small arrows produced a change in the maximum amplitude of the TRH response. After several minutes of wash the same oocyte was bathed in half  $[\text{Cl}^-]_o$  (59.8 mM) solution. B shows the effect of the addition of TRH (1  $\mu$ M) in this half  $[\text{Cl}^-]_o$  on the maximum amplitude of the current at the different holding potentials indicated by the small arrows.

A



B

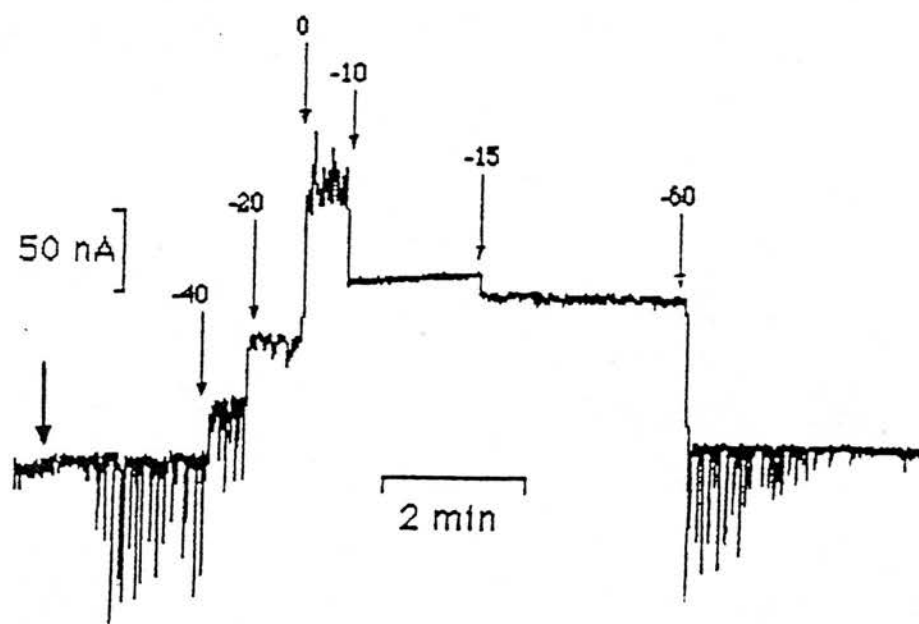
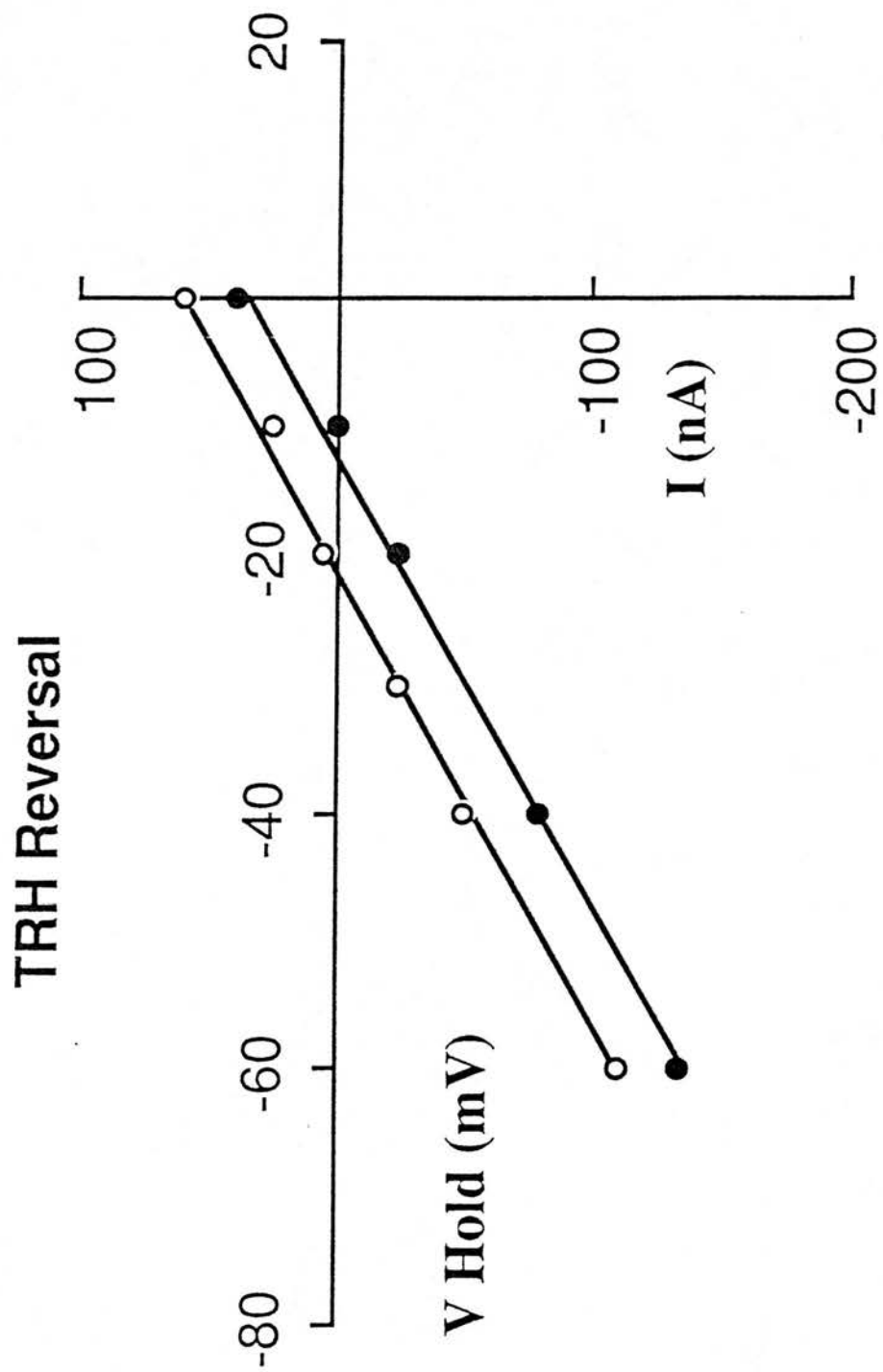


Figure. 6.4. Current-voltage (I-V) relation of the response induced by TRH

The observed maximum amplitude of current fluctuations induced by TRH were plotted against the holding potential. Open and filled circles, obtained from the same oocyte, were recorded in normal  $[\text{Cl}^-]_o$  (119.6 mM) and in half  $[\text{Cl}^-]_o$  (59.8 mM) solutions, respectively. The lines were fitted by linear regression analysis.



where the fitted line crossed the axis of the membrane potential  $V_m$  (the zero-current potential).

Dependence of the TRH-induced current on  $Cl^-$  ions was also demonstrated by changing the external concentration of  $Cl^-$ . An example of the effect of changing the external concentration of  $Cl^-$  on the TRH response of an oocyte is shown in Figure 6.3 B. The concentration of  $Cl^-$  was reduced to half (59.8 mM) by substituting the impermeant anion methanesulphonate for  $Cl^-$ , the subsequent application of TRH (1  $\mu$ M) elicited the usual inward current fluctuations, but upon changing the holding potential, the current flow became minimal at a holding potential of -10 mV. The linear portion of the I-V relationship in half  $Cl^-$  was plotted against the holding potential (filled circles in Fig. 6.4). The fitted dashed line ( $r = 0.998$ ) showed a parallel shift and the calculated reversal potential was -11 mV. The mean reversal potentials for currents induced by TRH recorded from the same oocytes in normal and half  $Cl^-$  solutions were  $-22.5 \pm 1.5$  mV and  $-8.0 \pm 3.0$  mV ( $n=3$ ), respectively. Therefore the average depolarizing shift of the reversal potential by halving the concentration of  $Cl^-$  was 14.5 mV. This value is in a good agreement with the expected value of 17.5 mV given by the Nernst equation (mentioned previously).

#### **Dependence of the TRH Response on $Ca^{2+}$**

Since other exogenous receptors expressed in *Xenopus* oocytes have been shown to produce current responses and the GnRH response has been shown to be linked to  $Ca^{2+}$ -dependent  $Cl^-$  channels in section 5, the dependence of the  $Cl^-$  channel activity on internal  $Ca^{2+}$  was examined for the TRH response. The TRH response was also inhibited by the blocker of intracellular  $Ca^{2+}$  release, TMB-8 (8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride) (Chiou & Malagodi, 1975) ( $n=3$ ). An example is shown in Fig. 6.5. The application of TRH (1  $\mu$ M) produced the expected inward current fluctuations. TMB-8 was applied at the concentration of 0.2 mM in the continued presence of 1  $\mu$ M TRH which produced a significant decrease in the membrane current. TMB-8 was then removed from the bathing solution, and the TRH response recovered.

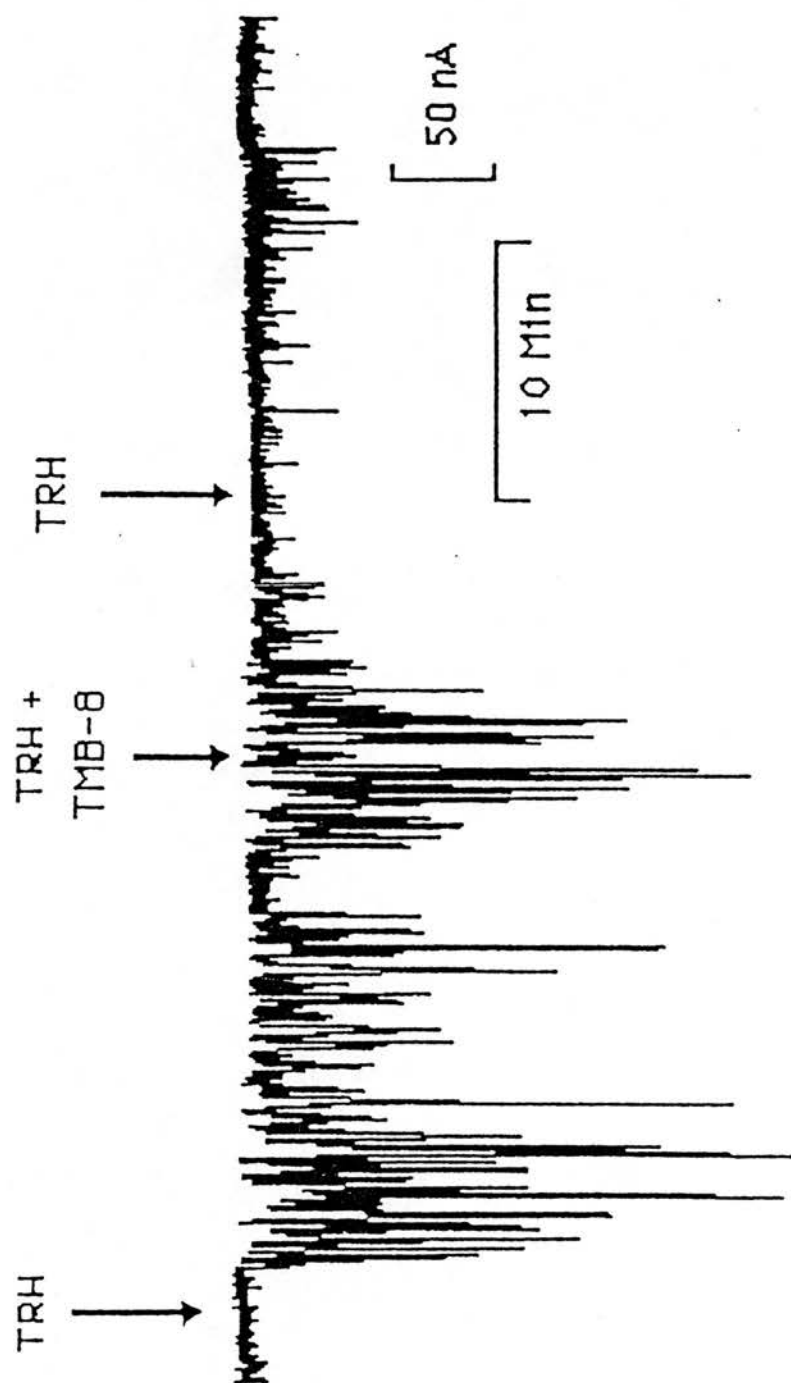
#### **Inhibition of the TRH response by pertussis toxin**

From the above results the TRH receptor would, like the GnRH receptor, appear to be linked to a second messenger system which releases intracellular calcium, it is probable that this involves the activation of a G-protein linked to

Figure.6 5. Suppression of the TRH response by a blocker of intracellular  $\text{Ca}^{2+}$  release, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8).

TRH (1 $\mu\text{M}$ ) was firstly applied to an oocyte and an inward current response elicited. TMB-8 (0.2 mM) was added to the cell in the continued presence of TRH and the inward current was inhibited. The TRH response was elicited again when TMB-8 was removed from the bathing solution . The points of chemical applications are indicated by arrows.





phospholipase-C. It has been established that pertussis toxin (PTX) selectively catalyses the ADP ribosylation of the  $\alpha$  subunits G-proteins (Gilman, 1987). Therefore, the effect of pertussis toxin was studied in mRNA-injected oocytes. Pre-incubation with pertussis toxin (2 $\mu$ g/ml) for 18hr completely abolished the response to TRH (n=3). An example of the effect of treatment with pertussis toxin on the TRH response is shown in Figure 6.6 A. In this oocyte the application of TRH (1  $\mu$ M) produced no response. In contrast, oocytes from the same donor, which had not been treated with pertussis toxin (n=3) were responsive to TRH. An example of such a control oocyte is shown in Figure 6.6 B, where the application of TRH (1  $\mu$ M) produces the usual inward current fluctuations.

### Fractionation of the mRNA

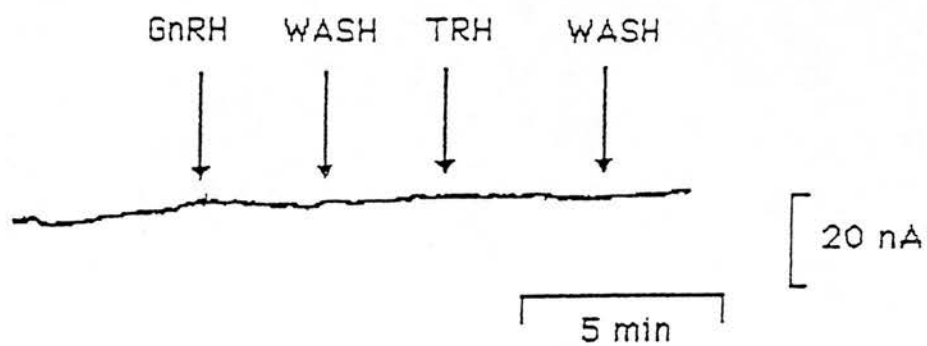
To determine the size of the RNA species encoding the TRH receptor, size fractionation of total RNA derived from rat anterior pituitary glands was carried out on NaDodSO<sub>4</sub> sucrose-density gradients. These size-selected fractions were injected separately into oocytes. Current responses were evoked by the application of TRH to oocytes injected with fractions eluting around the 18 S peak. An example of such an oocyte (injected with fractions 11-15) is shown in figure 6.7 A, where the application of TRH (1 $\mu$ M) produces current fluctuations. The size-fractionation of RNA on a sucrose density gradient is shown in Fig. 6.7 B. As responses were obtained from RNA fractions around 18 S, the mRNA's encoding for the TRH receptor are around 3,000 base-pairs in size.

### DISCUSSION

The results presented above indicate that the responses to TRH measured in *Xenopus* oocytes injected with rat pituitary mRNA, as with the response to GnRH, were mainly dependent on the activity of Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels. The TRH response was inhibited by the Cl<sup>-</sup> channel blockers 9-AC and DIDS. The latency of the TRH response was shorter than that of the GnRH response and the amplitude was larger than that for GnRH. This finding may reflect a difference in the number of TRH receptors. It has been demonstrated that the success-rate for the expression of the TRH receptor is greater than the expression of the GnRH receptor. A direct relationship has been shown between the dose of TRH administered and the amplitude of the Cl<sup>-</sup> current (Oron, Gillo, Straub & Gershengorn, 1988) and the amount of mRNA injected

Figure.6.6. The effect of pertussis toxin on the TRH response induced by injection of the *Xenopus* oocytes with rat pituitary poly (A)+ mRNA. (A) shows the effect of preincubation of an oocyte with pertussis toxin (2 $\mu$ g/ml) for 18 hr. The application of GnRH (1 $\mu$ M) and TRH (1 $\mu$ M) are indicated by the arrows. An oocyte from the same donor, also injected with the mRNA, but not treated with pertussis toxin is shown in B. The addition of GnRH and TRH (both at 1 $\mu$ M) elicited inward current fluctuations.

A



B

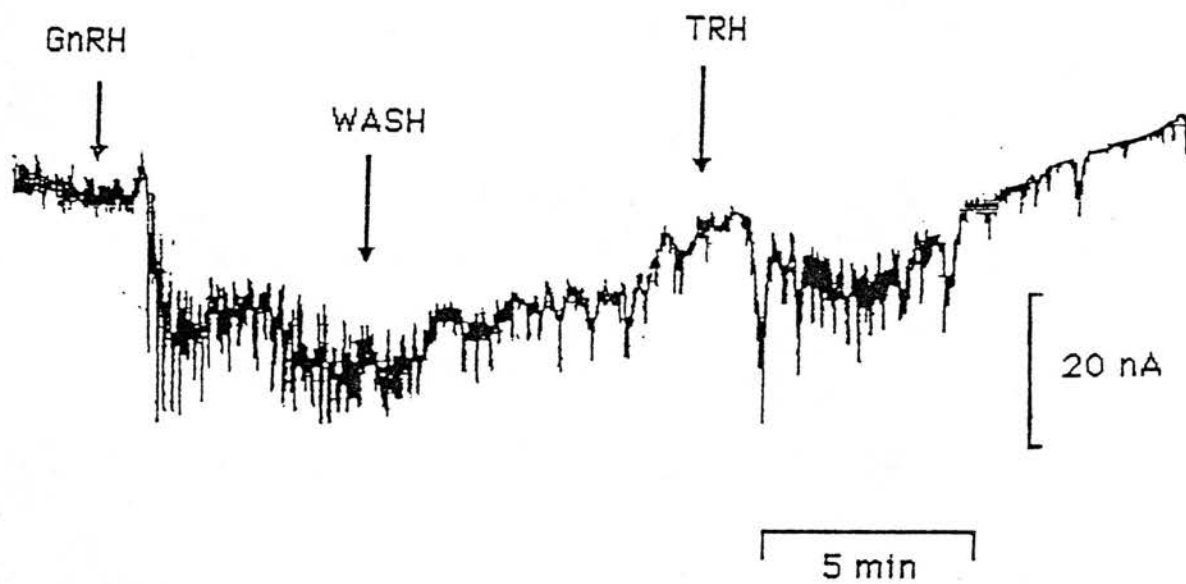
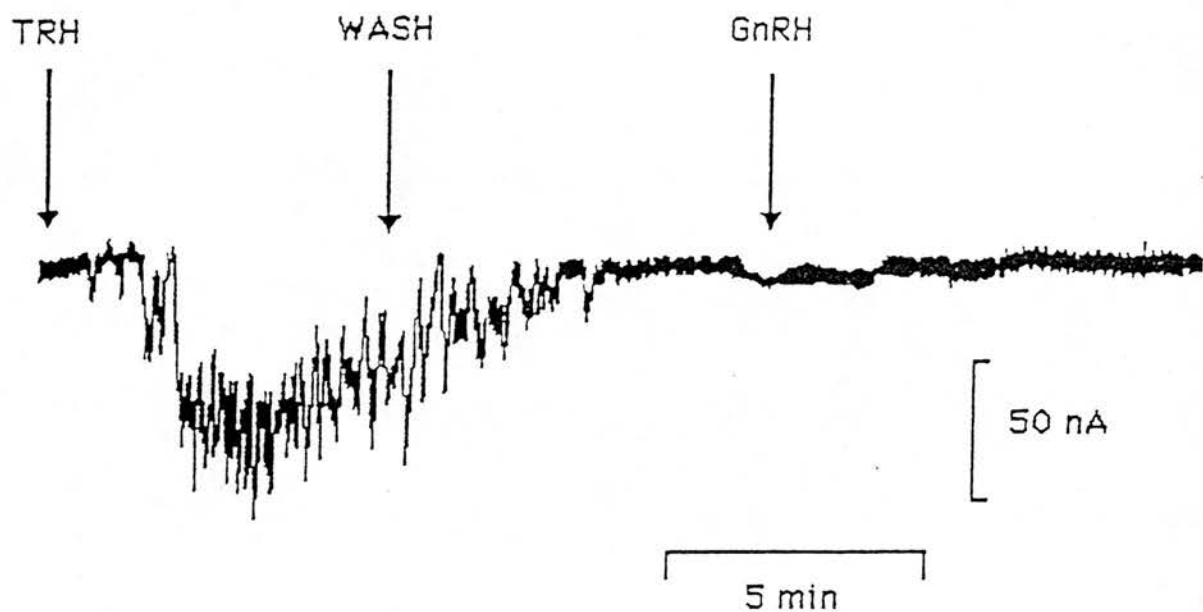


Figure. 6.7 The size fractionation of mRNA encoding the TRH receptor

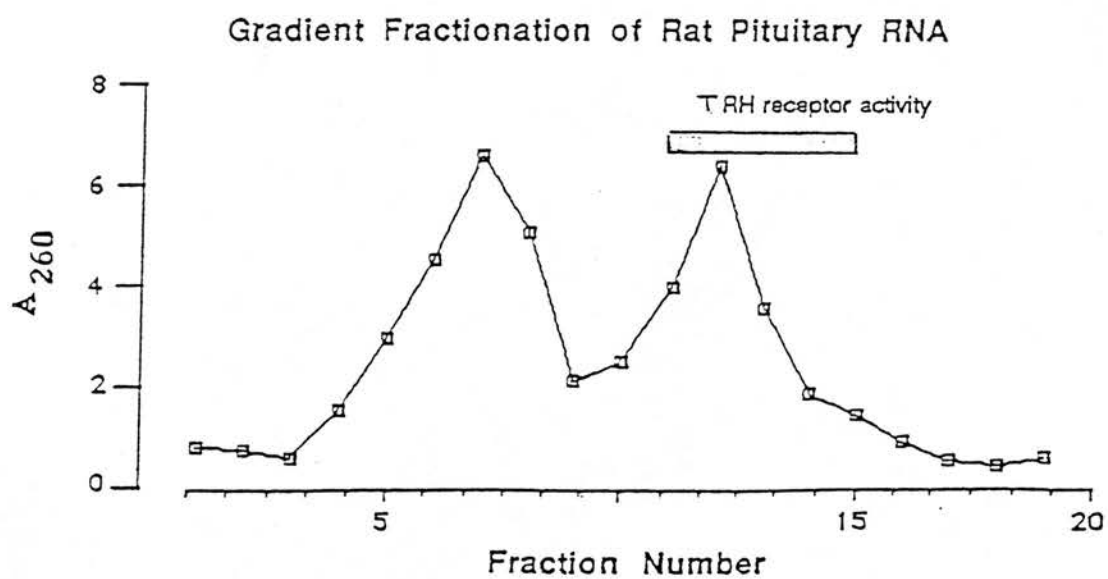
(A) shows the response to TRH (1 $\mu$ M) from an oocyte injected with fraction 11-15 of poly (A)+ mRNA. There was no response to GnRH (1 $\mu$ M) from this oocyte.

(B) Sucrose-density gradient of poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands. Individual fractions were tested by injection of 50ng into *Xenopus* oocytes for electrophysiological assay. The mRNA-injected oocytes were voltage clamped at -60 mV and exposed to 1  $\mu$ M TRH. Horizontal bar indicates the position of the mRNA fraction (fractions 11-15) which gave rise to positive responses to the peptides.

**A**



**B**



(Oron, Straub, Tractmann & Gershengorn, 1987). This is in keeping with the finding in GH<sub>3</sub> pituitary cells that the magnitude of the response to TRH is directly proportional to the density of the receptors (Ramsdell & Tashjian, 1986). The latency of the response to TRH in *Xenopus* oocytes has also been shown to be related to the number of receptors expressed (Straub, Oron, Gillo *et al.*, 1989). In this study they showed that the cause of this latency is a rate-limiting step in the activation of the Cl<sup>-</sup> channel. As injection of IP<sub>3</sub> or Ca<sup>2+</sup> produces a similar response with little delay, the rate limiting step would appear to occur before the release of IP<sub>3</sub>. As the latency is also dependent on receptor number it is attractive to postulate that the rate limiting step in the response to TRH (or GnRH) involves the receptor complex.

The reversal potential for the TRH response was approximately -22 mV, and this value agrees well with the reported reversal potential for Cl<sup>-</sup> in *Xenopus* oocytes (Dascal, Landau & Lass, 1984; Kusano, Miledi & Stinnakre, 1982; Busa & Nuccitelli, 1985). Further evidence for the dependency of the TRH response on Cl<sup>-</sup> was provided by the observed shift in the reversal potential with the change in concentration of external Cl<sup>-</sup>. The depolarizing shift of the reversal potential produced by halving the external Cl<sup>-</sup> concentration was 14.5 mV, similar to the value of 17.5 mV predicted by the Nernst equation for a Cl<sup>-</sup> electrode.

The activity of the Cl<sup>-</sup> channel responsible for the TRH response was dependent on internal Ca<sup>2+</sup>. TMB-8, a blocker of intracellular Ca<sup>2+</sup> release (Chiou & Malagodi, 1975), inhibited the TRH response. It is therefore likely that the TRH response is dependent on the mobilization of intracellular Ca<sup>2+</sup> for the activation of Cl<sup>-</sup> channels. As with GnRH, the direction of the current carried by Cl<sup>-</sup> ( $I_{Cl^-}$ ) in response to TRH can be described by the equation  $I_{Cl^-} = g_{Cl^-} (E - E_{Cl^-})$  where  $E$  and  $E_{Cl^-}$  indicate the membrane potential and the reversal potential for Cl<sup>-</sup>, respectively. Under voltage-clamp conditions when the holding potential is set at -60 mV inward current means an efflux of Cl<sup>-</sup>. This increase in chloride conductance would explain the depolarizing membrane-potential change observed with intracellular recording techniques in Section 3. Since the oocyte membrane potential shifts toward this equilibrium potential, this results in a membrane depolarization as the membrane becomes permeable to Cl<sup>-</sup> in response to TRH.

So far, exogenous receptors expressed in *Xenopus* oocytes for acetylcholine, serotonin, TRH, substance K and many more produce current

responses dependent on the activity of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Dascal, 1987 ; Snutch, 1988 for review). The  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance in *Xenopus* oocytes upon the addition of the ionophore A23187 was further classified into two components, a fast, transient and slow component (Boton, Dascal, Gillo & Lass, 1989). These two components have different  $\text{Ca}^{2+}$  dependence of activation and inactivation and different sensitivity to a  $\text{Cl}^-$  channel blocker. The  $\text{Cl}^-$  conductance in response to TRH in the present study resembles the slow component of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance in its time course and has a similar sensitivity to 9-AC. It is to be noted that the TRH response observed in the present study was not preceded by the fast response. Similar findings have been reported by Meyerhof, Morley, Schwartz & Richter, (1988) who used *Xenopus* oocytes injected with mRNA isolated from GH<sub>3</sub> cells of rat pituitary tumor origin. In contrast, Oron, Gillo, Straub & Gershengorn (1987) always observed a TRH response which consisted of a transient, rapid response followed by a long-lasting current with superimposed current fluctuations in *Xenopus* oocytes injected with mRNA isolated from GH<sub>3</sub> cells. That the absence of the fast component was not due to limitations of the recording equipment in our experiments, was tested by recording the fast response to serotonin (Julius, McDermott, Axel *et al.*, 1988) as shown in Section 4 (fig 4.3). The lack of the fast component of the TRH response in this study, may be due to the different source of mRNA. It seems likely that a cell-line expressing the TRH receptor would provide a source of mRNA in which the proportion of TRH message would be greater than that from the anterior pituitary, where, as shown previously (see section 4), a number of other receptors are being expressed. Hence the higher proportion of TRH receptors expressed from the cell-line may induce the fast component of the response. Indeed, it has been reported that the magnitude of the response to TRH in GH<sub>3</sub> cells is directly proportional to the density of the receptors expressed (Ramsdell & Tashjian, 1986). This possible explanation for a difference in the response following injection of pituitary mRNA in comparison with GH<sub>3</sub> RNA however, does not explain why two groups using mRNA from the same source (GH<sub>3</sub> cells) found different response profiles in the oocytes. It is interesting to note that in mouse thyrotropin tumor cells which express TRH receptors, TRH elicits a biphasic release of thyroid stimulating hormone (TSH). This response is elicited by a rapid IP<sub>3</sub>-linked  $\text{Ca}^{2+}$  rising phase followed by a slow sustained rise which is dependent on the external concentration of  $\text{Ca}^{2+}$ . However, when the number of TRH receptors is lowered by down regulation, only the slow component of the TSH response is



possible. Thus, there appears to be a minimal number of receptor-ligand complexes required to generate the IP<sub>3</sub>-linked fast component of the response (Winicov & Gershengorn, 1989).

In the pituitary lactotroph, TRH causes a rapid biphasic increase in the rate of release of stored prolactin (Gershengorn, 1986). The binding of TRH to its receptors stimulates breakdown of phosphoinositol bisphosphate (PIP<sub>2</sub>) and leads to an increase in the second messengers IP<sub>3</sub> and DAG. The IP<sub>3</sub> is believed to lead to release of intracellular calcium, accounting for a rapid rise in free Ca<sup>2+</sup>. Hence the responses to TRH are similar to those for GnRH (see Figure 5.12).

DAG is believed to activate protein kinase-C (PKC), which contributes to the second phase of hormone release by protein or ion channel phosphorylation (Drummond, Hughes, Luiz-Larrera & Joels, 1989). In *Xenopus* oocytes the activation of PKC appears to cause desensitization as in a study of the expressed serotonin and acetylcholine receptor responses, the activation of PKC caused a reduction in the response to these two agonists (Moran & Dascal, 1989). Thus, the desensitisation seen in the TRH response could be due to an effect of PKC which is endogenous to the oocyte.

Considerable evidence indicates that the TRH receptor interacts with an as yet unidentified G-protein to activate phospholipase-C (Aub, Frey, Sakura & Cote, 1986). Reports have indicated that there are two classes of G-protein involved in receptor-phospholipase-C coupling, which are either sensitive or insensitive to pertussis toxin in several cell lines. On the basis of this study with pertussis toxin the TRH receptor is linked to a G-protein which is pertussis toxin-sensitive and therefore the G-protein is similar to those found in human leukaemic (HL-60) and neutrophils (Volpi, Naccache *et al.*, 1985; Kikuchi, Kozawa, Kaibuchi *et al.*, 1986). There is evidence to suggest that pertussis toxin does not inhibit TRH action in GH3 tumor cells, which express TRH receptors (Wojcikiewicz, Kent & Fain, 1986; Hewlett & Gershengorn, 1986) and that the coupling of the TRH receptor in these cells to a guanine-nucleotide receptor, is susceptible to cholera toxin (Yajima, Akita & Saito, 1988). However, a further study has shown that neither pertussis or cholera toxin have an effect on the TRH response in GH3 cells (Martin, Bajjelih, Lucas & Kowalchuk, 1987). In pituitary cells, it has also been shown that the TRH response is not affected by pertussis toxin (Hewlett & Gershengorn, 1986).

The *Xenopus* oocyte has also been shown to contain endogenous G-proteins (Sadler, Maller & Cooper, 1984; Goodhardt, Ferry, Buscaglia *et al.*, 1984). The question which arises is whether the response observed in the *Xenopus* oocyte is due to the activation of an endogenous G-protein, or whether the G-protein is that found in the rat pituitary and is expressed along with the TRH receptor. As mentioned in the results section, a particular fraction of mRNA encodes the GnRH receptor and even after fractionation of the mRNA, the response is similar. Therefore, it seems likely, unless the G-protein has a similar molecular weight, that the TRH receptor is being expressed in isolation and is linked to an endogenous G-protein. Indeed the G-proteins are small molecules and would therefore not be in the fraction which has been shown to elicit a TRH response. The fractionation of the mRNA encoding the TRH receptor revealed that the size of the mRNA is around 3 kilo bases (kb). The TRH receptor mRNA has also been fractionated by another group, who found that the size was 4kb (Oron, Gillo, Straub & Gershengorn, 1987).

The present study has shown that the rat anterior pituitary gland mRNA encoding the TRH receptor can be translated in *Xenopus* oocytes. These receptors are able to couple up to G protein-linked second messenger systems present in *Xenopus* oocytes. The mechanism of this response depends on the activity of chloride channels which are endogenous in the oocytes (see Dascal, 1987 and Snutch, 1988 for review).

## SECTION 7

### A study of the intracellular calcium stores of the *Xenopus laevis* oocyte

The events leading up to the production of a chloride current following the activation of a receptor in the *Xenopus* oocyte membrane are not yet fully understood. It has been established that there is a release of calcium from intracellular stores, but the nature of these stores is still a matter of some debate. The chloride current response to acetylcholine (ACh) in *Xenopus* oocytes, first described by Kusano, Miledi & Stinnakre (1977) consisted of two components, a rapid, transient phase and a slow, long-lasting one which were dependent on intracellular calcium release. The aim of the following section is to study the intracellular store(s) which are responsible for these two current responses.

## RESULTS

### The characteristics of the Ionomycin response

Ionomycin is a  $\text{Ca}^{2+}$  ionophore (Liu & Harman, 1978) <sup>one of the actions of which is</sup> to release  $\text{Ca}^{2+}$  from intracellular stores (Hasford & Lakatta, 1987). We therefore used ionomycin to elucidate the mechanism of release of  $\text{Ca}^{2+}$  from intracellular stores in *Xenopus* oocytes. Endogenous and expressed receptors in *Xenopus* oocytes are coupled to endogenous  $\text{Cl}^-$  channels which are activated by the rise in the intracellular concentration of  $\text{Ca}^{2+}$ . This rise in intracellular  $\text{Ca}^{2+}$  is mainly caused by release from intracellular stores (for reviews see Dascal, 1987; Snutch, 1988). A response to externally applied ionomycin consisted of two components which were very similar to other responses such as those to serotonin and ACh receptors which have been expressed in the membrane of the *Xenopus* oocyte by the injection of mRNA (Julius *et al.*, 1989; Dascal & Landau, 1982; Dascal *et al.*, 1985). A representative example of the response to ionomycin is shown in Figure 7.1A. In this oocyte, the application of ionomycin ( $1\mu\text{M}$ ) induced a fast inward current response of 135nA followed by a slow component of 40nA. The only prominent difference in the ionomycin response from that of, for example serotonin, is that its slow component does not show current oscillations which are sometimes induced by serotonin and ACh (Julius

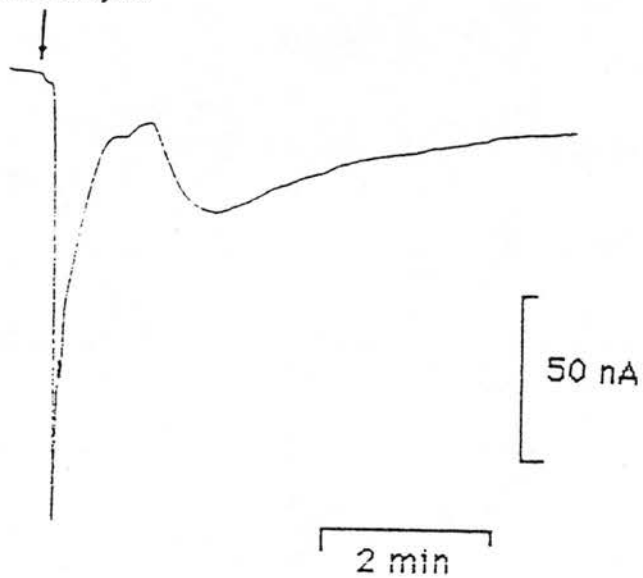
**Figure 7.1** Membrane current responses elicited by the ionophore ionomycin in *Xenopus* oocytes.

In (A) the application of ionomycin ( $1\mu\text{M}$ ) elicits an inward current response (downward deflection) which consists of two phases, a fast transient current and a slow, more prolonged one. The arrow indicates the point of perfusion with ionomycin.

(B) shows the effect of an increased dose of ionomycin ( $5\mu\text{M}$ ) on the membrane current.

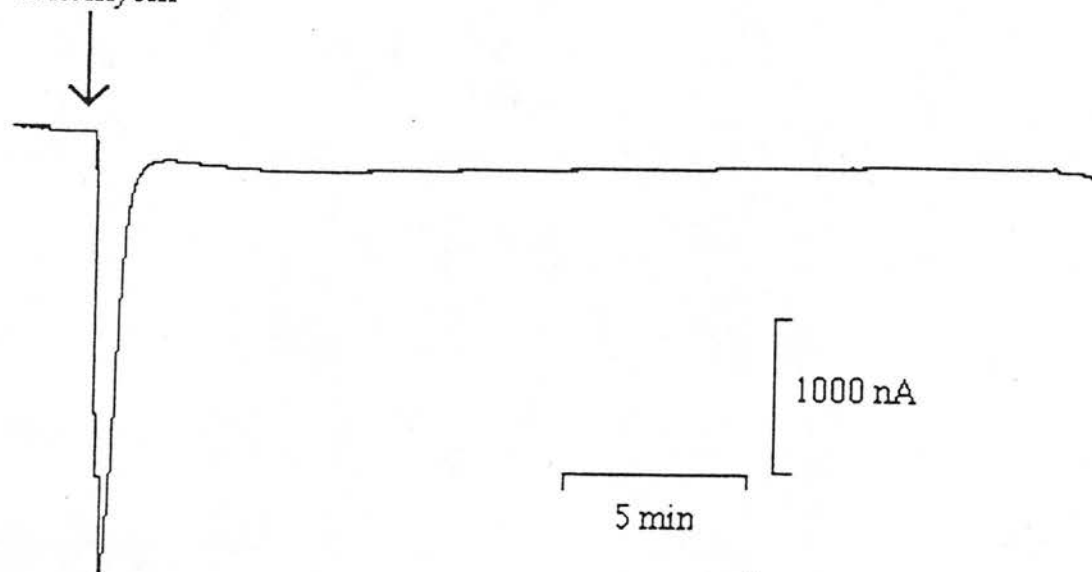
A

1  $\mu$ M Ionomycin



B

5  $\mu$ M  
ionomycin



*et al.*, 1989; Dascal & Landau, 1982). The response to ionomycin had a reversal potential of around -25mV and would therefore, as with the expressed receptor activation, appear to be due to the activation of chloride channels.

The ionomycin response occurred in a dose-dependent manner. When the concentration was 1  $\mu$ M, the amplitude of the fast response did not exceed 1000 nA. At higher concentrations of ionomycin, eg. 5  $\mu$ M, the response became larger as shown in Fig. 7.1B. The mean values of the maximum amplitude of the fast component were  $80 \pm 50$  nA (0.5  $\mu$ M; n=5),  $330 \pm 250$  nA (1  $\mu$ M; n=23), and  $3000 \pm 500$  nA (5  $\mu$ M; n=5). The amplitude of the second component was more variable, particularly from one frog to another. The slow component of the ionomycin response was less variable from oocytes of the same donor, hence for the subsequent studies oocytes from a single donor are used to study the effect of various manipulations of the oocytes on the slow component of the response.

### **Dependence of the ionomycin response on the intracellular $\text{Ca}^{2+}$ concentration**

The intracellular  $\text{Ca}^{2+}$  was chelated by a pH-insensitive specific chelator for  $\text{Ca}^{2+}$ , BAPTA (bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid). The acetoxymethyl ester of BAPTA (BAPTA/AM) is membrane permeant, and when it penetrates the cell membrane, it is cleaved by cytoplasmic esterases to yield free BAPTA and chelate intracellular free  $\text{Ca}^{2+}$ ; de-esterified BAPTA is membrane impermeant and locked within the cell (Tsien, 1981). Since no significant differences were observed in the chelating effect of intracellular free  $\text{Ca}^{2+}$  by externally applied BAPTA/AM by the presence or absence of  $\text{Ca}^{2+}$  in the bathing medium (Barritt & Lee, 1985), oocytes were treated with BAPTA/AM in standard solution; oocyte membranes became too leaky to record stable and reliable records in the absence of external  $\text{Ca}^{2+}$ .

Ionomycin (1  $\mu$ M) was applied to oocytes which had been preincubated with 1 or 10  $\mu$ M BAPTA/AM for 1-3 hr. At 1  $\mu$ M, BAPTA/AM did not show significant suppression of the ionomycin response (n=5). In contrast, when the concentration of BAPTA/AM was increased to 10  $\mu$ M, the fast component of the ionomycin response was completely blocked by BAPTA (n=3). An example of the effect of pre-treatment with BAPTA/AM is shown in Figure 7.2A. The mean value of the maximum amplitude of the slow component was reduced to  $11.7 \pm 2.9$  nA (n=3). The mean values of the maximum amplitude of the fast and slow components in control oocytes obtained from the same frog were  $110.0 \pm 63.8$  nA and  $29.7 \pm 15.4$  nA, respectively (n=3). A representative example of a

**Figure 7.2** The effect of the internal  $\text{Ca}^{2+}$  chelator BAPTA/AM on the response to ionomycin.

In (A) an oocyte has been treated with BAPTA/AM ( $10\mu\text{M}$ ) for 2 hours. A subsequent application of ionomycin produces only a reduced slow membrane current response.

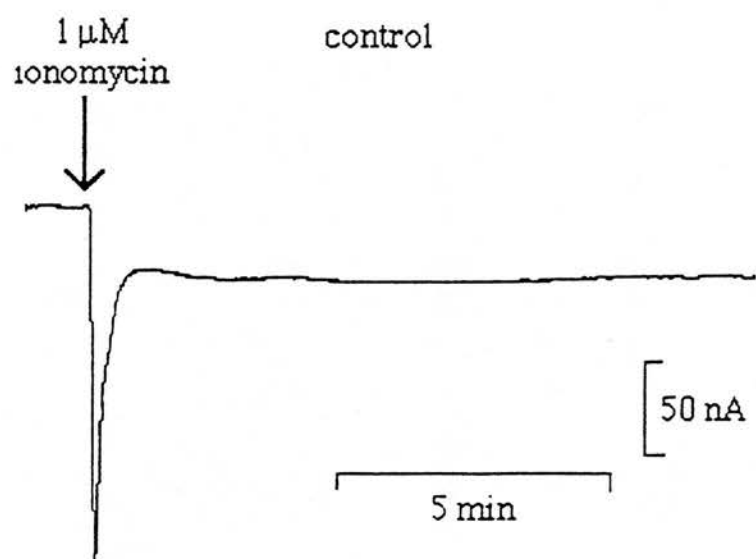
In (B) an oocyte from the same donor frog shows the normal response to ionomycin ( $1\mu\text{M}$ ). The calibrations apply to both records.

A

In the presence of 10  $\mu$ M BAPTA/AM



B





control response to ionomycin from the same donor frog is shown in Figure 7.2 B. The statistical Mann-Whitney two-tailed U test shows that there was a tendency that the slow component was suppressed by BAPTA/AM but it was not statistically significant. It is concluded that the ionomycin response is dependent on the rise in the intracellular  $\text{Ca}^{2+}$  concentration and that the fast component of the ionomycin response is more sensitive to BAPTA than the slow component.

### **The dependence of the response to ionomycin on extracellular $\text{Ca}^{2+}$**

The ionomycin response could be elicited in  $\text{Ca}^{2+}$ -free bathing medium (Fig. 7.3); In figure 7.3 A, external  $\text{Ca}^{2+}$  was chelated by 2 mM EGTA. Oocyte membranes became too leaky to be voltage clamped when external  $\text{Ca}^{2+}$  was chelated by EGTA. Therefore, divalent cations; in this case 10 mM  $\text{Mg}^{2+}$  was used to replace  $\text{Ca}^{2+}$ . As shown in Figure 7.3 B the amplitude of the fast component did not significantly change in the absence of external  $\text{Ca}^{2+}$  in comparison with the control response to ionomycin (Figure 7.3A). The second slow component appeared to become smaller in size but this trend was not statistically significant. The average maximum amplitudes of the fast component in oocytes from this donor were  $180 \pm 100$  nA ( $n=5$ ) and  $210 \pm 87$  nA ( $n=5$ ) in standard solution and  $\text{Ca}^{2+}$ -free, 2 mM EGTA solution, respectively.

When  $\text{Ca}^{2+}$  was replaced with equimolar amounts of other divalent cations,  $\text{Mn}^{2+}$  ( $n=5$ ) and  $\text{Ba}^{2+}$  ( $n=5$ ) similar results were obtained. Representative examples of the response to ionomycin when the external  $\text{Ca}^{2+}$  had been replaced with  $\text{Mn}^{2+}$  and  $\text{Ba}^{2+}$  are shown in Figures 7.3 C & D respectively. There was no significant change in the fast component of the response, but this did appear to become larger. The slow component appeared to be reduced, but once again this was not statistically significant. These results indicate that the fast component is not dependent on external  $\text{Ca}^{2+}$ , but that the slow component would appear to be somewhat dependent on the entry of  $\text{Ca}^{2+}$  from the extracellular solution.

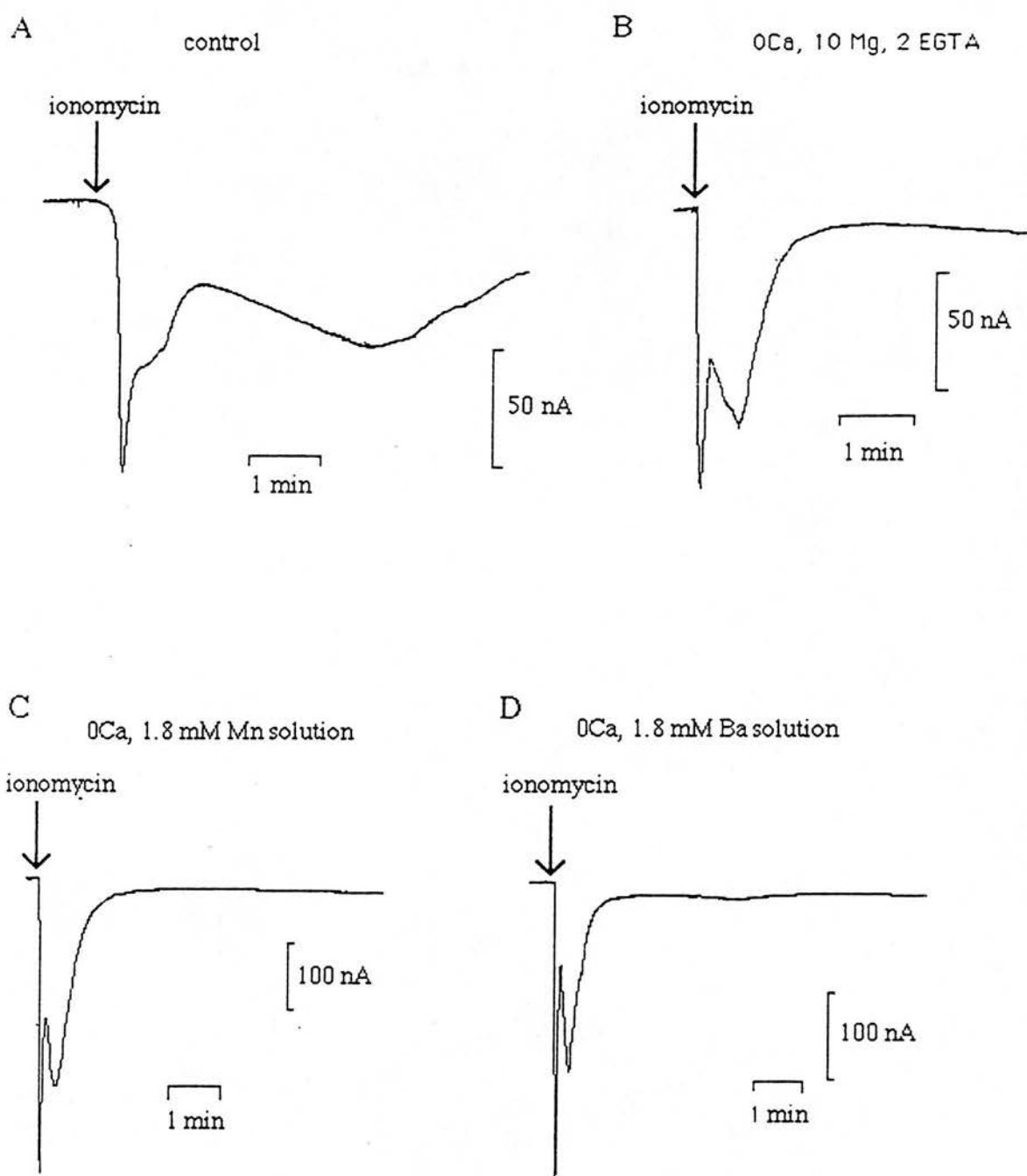
### **The involvement of an $\text{IP}_3$ -sensitive store in the response to ionomycin.**

The mechanism for the activation of a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current by ionomycin response did not seem to involve guanine nucleotide-binding regulatory proteins (G proteins), because ionomycin responses were still observed in oocytes which had been treated with 2  $\mu\text{g/ml}$  PTX for 18 h; this PTX treatment is sufficient to

**Figure 7.3** The effect of removing  $\text{Ca}^{2+}$  from the external bathing solution on the response to ionomycin.

(A) shows a representative example of the response to  $1\mu\text{M}$  ionomycin in an oocyte.

(B,C & D) show the effect of removing the external  $\text{Ca}^{2+}$  on the response to ionomycin ( $1\mu\text{M}$ ) . In (B) external  $\text{Ca}^{2+}$  has been removed and chelated with EGTA and  $10\text{mM}$   $\text{Mg}^{2+}$  has been added to aid membrane stability. In (C)  $1.8\text{mM}$   $\text{Mn}^{2+}$  has replaced  $\text{Ca}^{2+}$  and in (D)  $1.8\text{mM}$   $\text{Ba}^{2+}$  replaced  $\text{Ca}^{2+}$ . All cells were exposed to  $1\mu\text{M}$  ionomycin.



block the responses to GnRH and TRH in *Xenopus* oocytes, as mentioned in sections 5 & 6. Thus the ionomycin response does not appear to activate the G-protein known to cause a rise in intracellular calcium.

To examine whether or not ionomycin acts via second messenger <sup>5</sup>, a blocker of IP<sub>3</sub>-induced <sup>and Ca<sup>2+</sup>-induced</sup> Ca<sup>2+</sup> release, ruthenium red (Volpe *et al.*, 1985; Adunyah & Dean, 1986) was tested on the *Xenopus* oocyte. Ruthenium red (1-20  $\mu$ M) could not block the ionomycin response ( $n=6$ ).

Ionomycin does, however, act on the same store as IP<sub>3</sub>, as is shown in figure 7.4. In figure 7.4 A, the application of ionomycin (5 $\mu$ M) induces a large inward current response, but a subsequent injection of IP<sub>3</sub> (50nl of 0.1 $\mu$ M) fails to elicit a response. The injection of IP<sub>3</sub> is known to cause a membrane response ordinarily. Thus the failure to elicit a response after a response to ionomycin has occurred, suggests that ionomycin and IP<sub>3</sub> act on the same intracellular Ca<sup>2+</sup> store and that the response to ionomycin has depleted this store of Ca<sup>2+</sup>. Similarly, as shown in Figure 7.4 B, the injection of IP<sub>3</sub> induces a membrane response, but if ionomycin (5 $\mu$ M) is applied in quick succession no membrane response is elicited to ionomycin. Thus it would appear that ionomycin and IP<sub>3</sub> act on the same Ca<sup>2+</sup> store.

### Involvement of the endoplasmic reticulum in Ca<sup>2+</sup> release

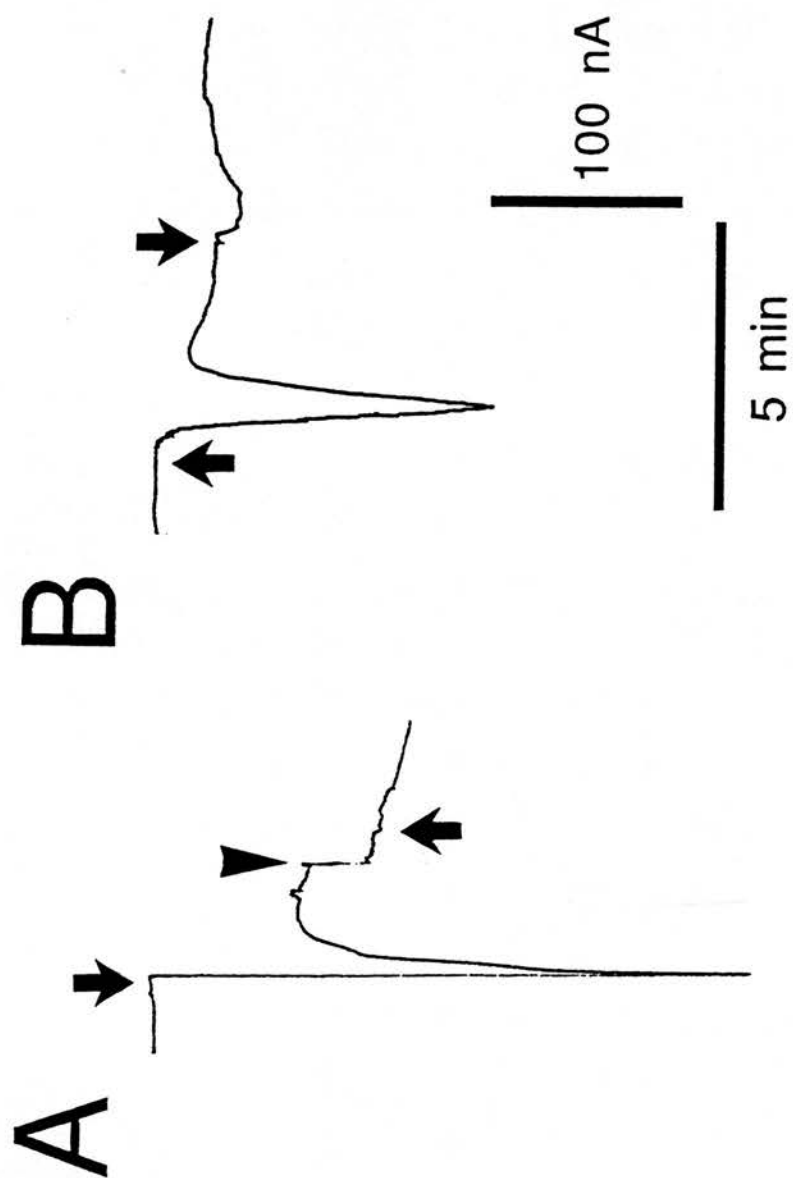
Caffeine (1,3,7-trimethylxanthine) is known to release Ca<sup>2+</sup> from intracellular stores in various types of preparations, for example from the sarcoplasmic reticulum (SR) in skeletal muscle cells (Endo, 1975) and in smooth muscle cells (Deth & Casteels, 1977; Deth & van Breemen, 1977; Leijten & van Breemen, 1984), and from the endoplasmic reticulum in sympathetic ganglion cells (Kuba, 1980). In the present study, defolliculated and uninjected *Xenopus* oocytes were exposed to 10-20 mM caffeine, and no significant current was induced ( $n=8$ ). An example of an oocyte being exposed to caffeine (10mM) is shown in Figure 7.5 A.

Effect of oxalic acid on the ionomycin response was also tested. Oxalic acid is a stimulator of Ca<sup>2+</sup> uptake by the endoplasmic reticulum (Baun *et al.*, 1988). Since oxalic acid forms insoluble calcium-oxalic acid complex in the presence of Ca<sup>2+</sup>, an equimolar amount of Mn<sup>2+</sup> substituted for Ca<sup>2+</sup> in the bathing solution; the absence of external calcium does not affect the ionomycin response. Although there is a report that high concentration (18 mM) of Mn<sup>2+</sup> potentiated the ACh-induced currents in *Xenopus* oocytes (Dascal, Gillo & Lass, 1985), the

**Figure 7.4** The effect of ionomycin on the response to IP3.

In (A), the initial downward pointing arrow indicates the application of 5 $\mu$ M ionomycin. The following arrow denotes the insertion of the IP3-injecting microelectrode into the oocyte and the upward pointing arrow shows the injection of 50nl of 0.1 $\mu$ M IP3 into the cell.

In (B), the upward-pointing arrow indicates the injection of IP3 (50 nl of 0.1 $\mu$ M). The downward pointing arrow indicates the addition of ionomycin to the cell (5 $\mu$ M).



**Figure 7.5.** The effect of known stimulators of the endoplasmic reticulum

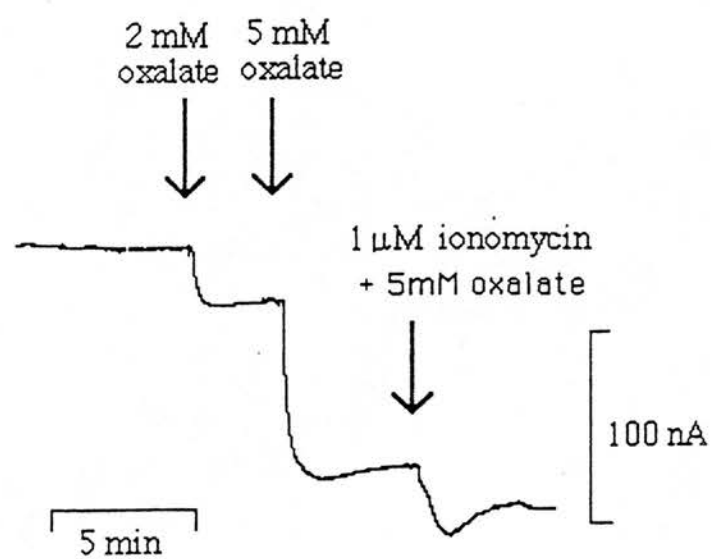
In (A) caffeine (10mM), a known stimulator of  $\text{Ca}^{2+}$  release from the endoplasmic reticulum caused no change in the membrane current of the cell.

In (B) oxalate, a known stimulator of  $\text{Ca}^{2+}$  uptake by the endoplasmic reticulum caused an inhibition in the membrane current induced by ionomycin.

A



B





concentration (1.8 mM) used in the present study did not significantly affect the amplitude of the ionomycin response (as mentioned previously).

31. Oxalic acid on its own reversibly induced a steady inward current in *Xenopus* oocytes in a dose-dependent manner (Fig. 7.5 B). The concentration of oxalic acid was gradually increased from 1 to 5 mM, and the inward current became larger in amplitude. The original current level was recovered upon removal of oxalic acid from the bathing solution. Since oxalic acid (1-5 mM) could not exert any effect on the ionomycin response when it was applied to oocytes together with ionomycin, oocytes were preincubated for 5-15 min with oxalic acid prior to the application of ionomycin with the same concentration of oxalic acid. At 1 mM oxalic acid, no significant change was observed in the ionomycin response whereas the ionomycin response was inhibited by 5 mM oxalic acid (n=5). An example of the effect of oxalic acid on a *Xenopus* oocyte and on the response elicited by ionomycin is shown in Figure 7.4 B.

### Effects of mitochondrial inhibitors on the ionomycin response

Mitochondria are described as intracellular  $\text{Ca}^{2+}$  stores in some preparations, as addition, release and uptake of  $\text{Ca}^{2+}$  by mitochondria has been demonstrated in an isolated mitochondrial preparation (Carafoli *et al.*, 1966). Mitochondria are also an important energy source as they produce ATP. To study the involvement of mitochondria in the response to ionomycin, the effects of the mitochondrial inhibitors 2,4-dinitrophenol (DNP)(Yada *et al.*, 1986) and oligomycin (Streb & Schulz, 1983) were examined on the ionomycin response.

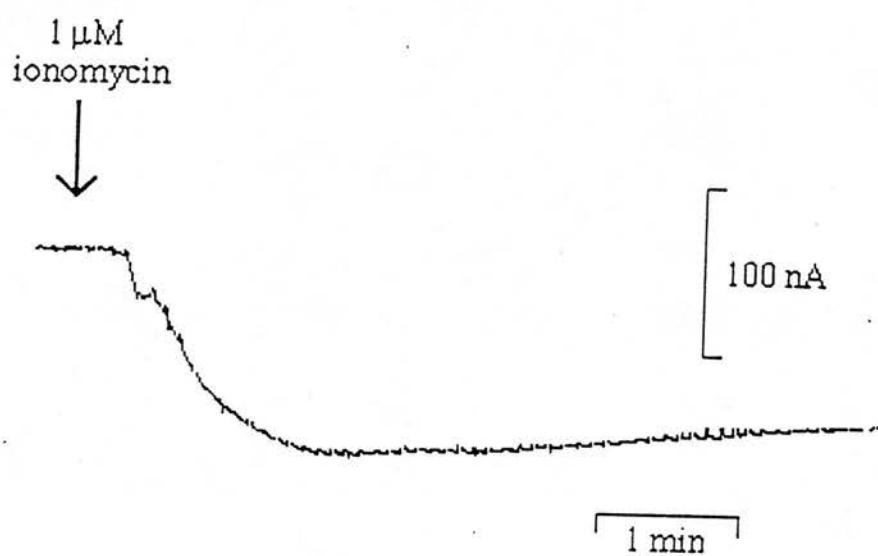
When 2,4-dinitrophenol (DNP; 0.1 or 0.5 mM) and ionomycin (1  $\mu\text{M}$ ) were simultaneously applied to oocytes, no significant changes in the ionomycin response were detected. In contrast, a blocking effect on the ionomycin response was observed when oocytes were preincubated with DNP for longer than 5 min (between 5 min and 2 hr), indicating that the blocking action of DNP on the ionomycin response is not instantaneous and that it takes time for DNP to penetrate the cell membrane to exert its inhibitory action in the cell. A slow inward current was observed when 1  $\mu\text{M}$  ionomycin and 0.1 mM DNP were simultaneously applied to an oocyte which had been preincubated with 0.1 mM DNP for 18 min (Figure 7.6 A). Thus, the fast component was completely blocked by 0.1 mM DNP whereas the slow component was only slightly reduced at this concentration. Similar complete block of the fast component and the partial block of the slow component were observed in 3 out of 6 oocytes; complete block of both components were seen in the other 3 oocytes. In

**Figure 7.6** The effect of the metabolic inhibitor 2,4-dinitrophenol (DNP) on the response to ionomycin

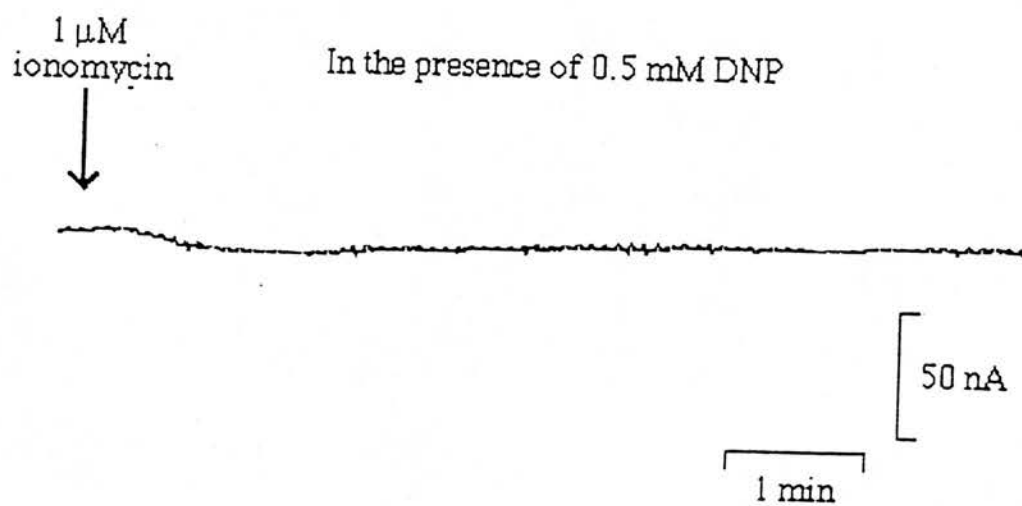
In (A) pretreatment with 0.1 mM DNP causes an inhibition of the fast response to ionomycin (1 $\mu$ M)

In (B) pretreatment with 0.5 mM DNP causes an inhibition of the fast and slow responses to ionomycin (1 $\mu$ M)

A In the presence of 0.1 mM DNP



B



contrast, when oocytes were preincubated with a higher concentration (0.5 mM) of DNP, both fast and slow components were completely blocked as displayed in Fig. 7.6 B. The oocyte was preincubated with 0.5 mM DNP for 14 min. It is concluded that the transient fast component of the ionomycin response was more sensitive to DNP than the slow component.

The effect of another mitochondrial inhibitor, oligomycin, on the ionomycin response was also tested (Streb & Schulz, 1983). The mean amplitude of the fast component of the inward current responses induced by 1  $\mu$ M ionomycin was  $326.7 \pm 163.5$  nA (n=6) in control oocytes. An example of the effect of oligomycin is shown in figure 7.7. The membrane current elicited by ionomycin (1  $\mu$ M) in oocytes which were preincubated with 5  $\mu$ M oligomycin for 1-3 hrs was  $159.5 \pm 98.8$  nA (n=10). Significant suppression of the ionomycin response was observed by oligomycin according to the Mann-Whitney U test (two-tailed; P=0.05).

#### **Effect of ATP on the action of a mitochondrial inhibitor**

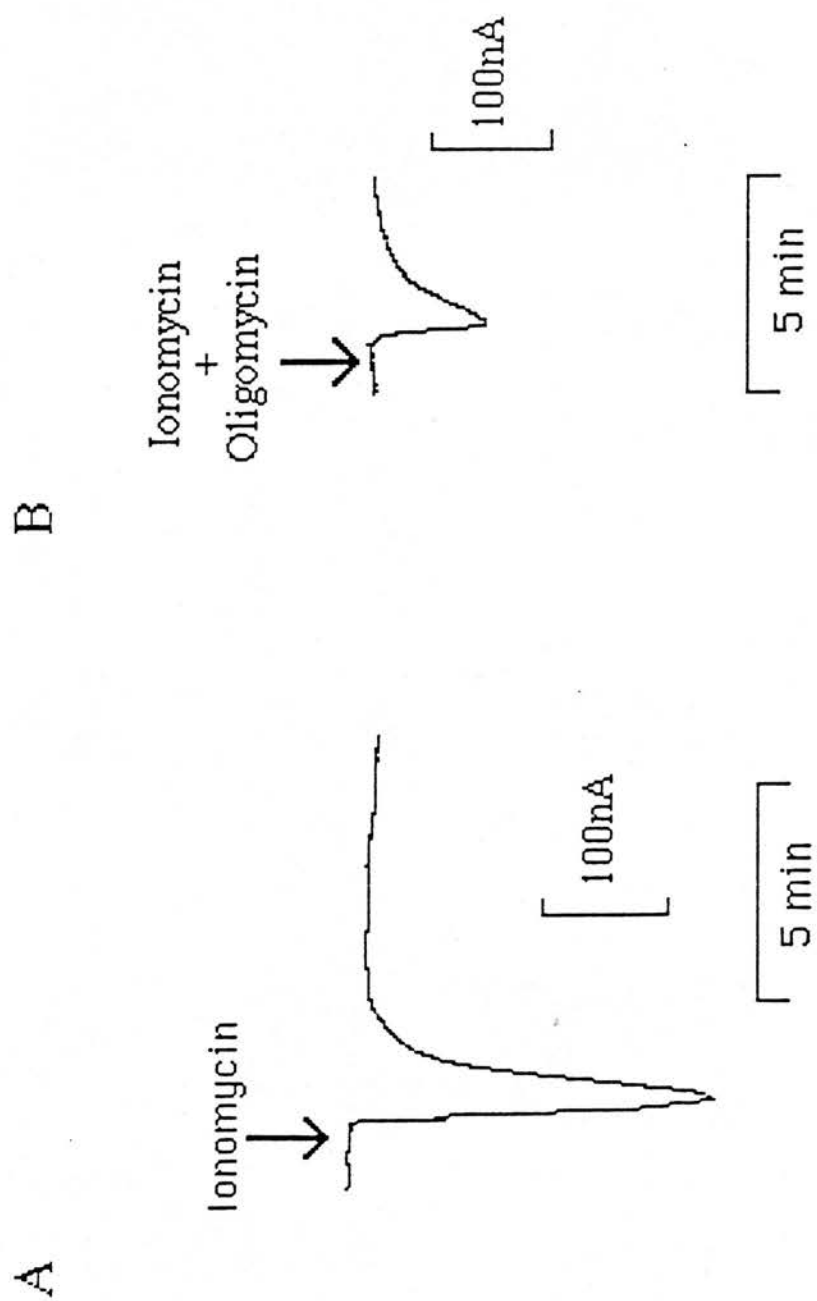
Although significant inhibition of the ionomycin response was observed by the application of mitochondrial inhibitors, DNP and oligomycin, there is a possibility that the inhibitory effect on the ionomycin response is due to some other ATP (adenosine 5'-triphosphate)-dependent mechanisms rather than by a direct effect by mitochondria. We therefore examined the effect of DNP on the ionomycin response in the presence of external ATP which is reported to influence a variety of biological processes including mobilization of intracellular bound  $\text{Ca}^{2+}$  (see Gordon, 1986), assuming that ATP can enter the cell.

The application of ATP (1mM) evoked a biphasic response, inward current followed by outward current, in follicular oocytes as has been previously reported (Lotan *et al.*, 1986) (Figure 7.8 A). Both inward and outward currents were blocked by DNP in follicular and denuded oocytes. The effect of DNP on the ATP response was dose- and time-dependent. Preincubation period longer than 20 min was necessary for DNP to be effective. A partial block of the ATP response was obtained by 0.1 mM DNP, and a complete block by 0.5 mM DNP (Figure 7.8 B). As mentioned previously, the ionomycin response could be completely inhibited by 0.5 mM DNP, but a partial recovery of the ionomycin response was observed in an oocyte treated with DNP (0.5mM) when the oocyte was also incubated with ATP, as is shown in Figure 7.8 C. This suggests that the events elicited by ionomycin involve some sort of active process which require ATP and that the major source of ATP is the mitochondria.

**Figure 7.7** The effect of the metabolic inhibitor oligomycin on the response to ionomycin

In (A) a control oocyte shows an inward current response to ionomycin ( $1\mu\text{M}$ ).

In (B) pretreatment with oligomycin ( $5\mu\text{M}$ ) for 2 hours causes a suppression of the response to ionomycin ( $1\mu\text{M}$ ).



**Figure 7.8** The effect of ATP on the inhibitory action of DNP on the response to ionomycin.

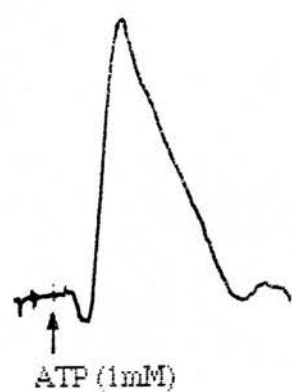
(A) shows the membrane current induced by the application of ATP (1mM) to a *Xenopus* oocyte.

(B) shows the effect of pretreatment of an oocyte with DNP (0.5 mM) on the response to ATP (1mM).

In (C) an oocyte which has been pretreated with DNP (0.5 mM) elicits a response to ionomycin (1 $\mu$ M) in the presence of ATP (1mM)

In (D) a control oocyte shows an inward current response to ionomycin (1 $\mu$ M).

A



B

Pre-incubated with DNP 0.5 mM

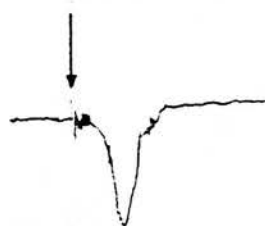
0.5 mM DNP  
+ 1mM ATP



C

Pre-incubated with DNP 0.5 mM

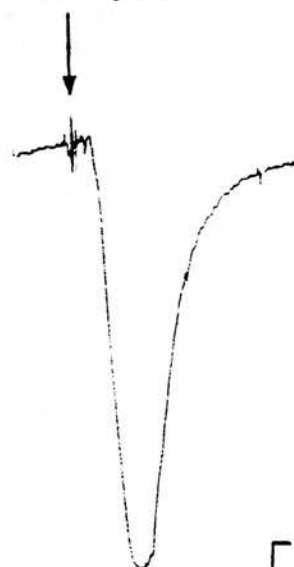
0.5 mM DNP  
+ 1mM ATP  
+ 1 $\mu$ M Ionomycin



D

CONTROL

1 $\mu$ M Ionomycin



2 min

50 nA



## DISCUSSION

Ionomycin, a  $\text{Ca}^{2+}$  ionophore, is reported to release  $\text{Ca}^{2+}$  from intracellular stores in rat cardiac myocytes (Hansford & Lakatta, 1987). In the present study the application of ionomycin elicited an inward  $\text{Cl}^-$  current response which consisted of two phases, a fast transient and a slow component. A  $\text{Ca}^{2+}$  ionophore A23187 has been used previously to study the  $\text{Cl}^-$ -current response in *Xenopus laevis* oocytes (Boton *et al.*, 1989). The application of A23187 by itself caused only a slow inward current response which was thought to reflect a slow entry into the cell. Therefore, in the study by Boton *et al.* (1989), *Xenopus* oocytes were permeabilized to  $\text{Ca}^{2+}$  with the  $\text{Ca}^{2+}$  ionophore A23187, and inward  $\text{Cl}^-$  current were evoked by subsequent applications of different external  $\text{Ca}^{2+}$  concentrations. When  $[\text{Ca}^{2+}]_o$  was higher than 2 mM, the  $\text{Cl}^-$  current consisted of two distinct kinetic components, a fast and transient one and a slow one, whereas when  $[\text{Ca}^{2+}]_o$  was between 0.1 and 1 mM, the current showed only a slow component. Thus the response to ionomycin resembles that of other ionophores, but the molecule would appear to move more rapidly through the oocyte membrane as it causes a rapid change in the membrane current.

The nature of the response to an ionophore has not been explored in detail previously. In the present study, pertussis toxin which is known to inhibit the action of the G-protein known to cause a release of the second messenger IP<sub>3</sub> (Moriarty *et al.*, 1990) had no effect on the response to ionomycin. In addition, ruthenium red which has been reported to block IP<sub>3</sub>-induced  $\text{Ca}^{2+}$  release from skeletal SR vesicles <sup>and skinned fibers</sup> (Volpe *et al.*, 1985) <sup>and  $\text{Ca}^{2+}$ -induced</sup> (Gillo *et al.*, 1986) and from human platelets (Adunyah & Dean, 1986) also had no effect on the response to ionomycin. Thus ionomycin does not cause the activation of a G-protein. However,

ionomycin and IP<sub>3</sub> are shown to act on the same type of  $\text{Ca}^{2+}$  store within the cell. Therefore the most likely explanation for the response to ionomycin is that it has a direct action on the intracellular stores, causing the release of  $\text{Ca}^{2+}$  and the activation of an inward chloride current. In a study by Gillo *et al.* (1987), submembranal injections of IP<sub>3</sub> evoked a two-component response, but when the injection pipette was deeply inserted into the oocyte, the fast component diminished and the slow component remained unchanged or even increased. The slow component was more sensitive to injected IP<sub>3</sub>. It is considered that  $\text{Ca}^{2+}$  stores possess specific receptors for IP<sub>3</sub>, resulting in rapid release of  $\text{Ca}^{2+}$  from the stores (see review by van Breemen & Saida, 1989). As the fast response to

ionomycin resembles that of intracellular injection of the second messenger IP3 it seem likely that the current induced by ionomycin is produced by release of stores which are sensitive to IP3.

Having established that ionomycin and IP3 act on the same intracellular  $\text{Ca}^{2+}$  stores, the next question which arises is, which organelle(s) form the  $\text{Ca}^{2+}$  stores. This subject has caused some debate. It has been shown, in permeabilised exocrine pancreatic cells, that IP3 releases  $\text{Ca}^{2+}$  from a non-mitochondrial store, since mitochondrial inhibitors did not affect the release of  $\text{Ca}^{2+}$  (Streb & Shultz, 1983) and also, it was shown that  $\text{Ca}^{2+}$  <sup>uptake</sup> was by some ATP-dependent store other than mitochondria (Streb *et al.*, 1983). This non-mitochondrial uptake <sup>of</sup>  $\text{Ca}^{2+}$  was completely dependent on  $\text{Mg}^{2+}$ , suggesting the action of  $\text{Mg}^{2+}$ -dependent ATP-ase, also, the uptake by this pool was stimulated by oxalate, which may imply that the store is the endoplasmic reticulum.

The *Xenopus* oocyte has several proposed intracellular calcium stores, these include; endoplasmic reticulum, mitochondria and the calciosome a specific organelle (Volpe *et al.*, 1988). According to electron probe X-ray microanalysis, mitochondria in situ contain only between 1 and 2 nM per mg of protein (Somlyo, 1985; Somlyo *et al.*, 1985b). The amount of  $\text{Ca}^{2+}$  therefore which can be mobilized is limited. Thus it does not seem likely that the main calcium store of the *Xenopus* oocyte are mitochondria. Endoplasmic reticulum is considered to be a major intracellular store of  $\text{Ca}^{2+}$  in nonmuscle cells (Somlyo *et al.*, 1985a), while SR to be that in smooth muscle cells (see review by van Breemen & Said, 1989) and skeletal and heart muscle cells. Therefore in the *Xenopus* oocyte, it seems likely that the major IP3-sensitive calcium store will be the endoplasmic reticulum. Indeed it has been shown that the *Xenopus* oocyte has extensive endoplasmic reticulum (Gardiner & Gray, 1983). The second messenger IP3 has been reported to release  $\text{Ca}^{2+}$  from endoplasmic reticulum (Berridge & Irvine, 1989) and more recently, the endoplasmic reticulum has been shown to contain the receptor for IP3 (Mignery, Sudhof, Takei & De Camilli, 1989). Further evidence comes from the use of oxalic acid, which is known to stimulate the ATP-dependent  $\text{Ca}^{2+}$  uptake by endoplasmic reticulum in the rat parotid microsomal membrane (Baum *et al.*, 1988). In the present study oxalic acid was shown to cause an inhibition of the response to ionomycin. This inhibition is possibly due to a buffering of the intracellular  $\text{Ca}^{2+}$  concentration which has already been shown to be important for the fast response to ionomycin. Such a finding lends further weight to the presence of the endoplasmic reticulum as a

Ca<sup>2+</sup> store. Further evidence for the presence of an endoplasmic reticulum calcium store was hoped to be obtained from the study of caffeine. Caffeine is known to cause the release of Ca<sup>2+</sup> from the endoplasmic reticulum. (see review by van Breemen & Saida, 1989). Caffeine (10-20 mM), however, was not effective in inducing any currents in *Xenopus* oocytes, suggesting that the endoplasmic reticulum is not the main Ca<sup>2+</sup> source or it is not sensitive to caffeine. Interestingly, caffeine failed in inducing release of Ca<sup>2+</sup> from stores in hamster oocytes (Georgiou *et al.*, 1988) and mouse fibroblasts (Okada *et al.*, 1982). Hamster oocytes are known to respond to intracellular injection of IP3 (Miyazaki, 1988). Thus it may be that some endoplasmic reticulum is insensitive to caffeine and this may be the case for the *Xenopus* oocyte.

The endoplasmic reticulum contains Ca<sup>2+</sup> ATPases, these ATPases mediate the process of Ca<sup>2+</sup> uptake (Constantin *et al.*, 1965; Carafoli, 1987). Mitochondria are known to produce ATP and therefore it was thought that mitochondria may play some role in the storage of intracellular Ca<sup>2+</sup> in the endoplasmic reticulum. From the results section it is clear that mitochondrial inhibitors such as oligomycin and DNP have an inhibitory effect on the ionomycin response. In cultured intestinal epithelial cells, release of Ca<sup>2+</sup> from intracellular stores was inhibited by mitochondrial inhibitors, KCN, NaN<sub>3</sub>, antimycin A, and DNP (Yada *et al.*, 1986). This finding may lead one to the conclusion that the calcium store responsible for the ionomycin response is mitochondria. However, the effect of DNP on the ionomycin response was partially restored by ATP. Since the ATP response was completely blocked by 0.5 mM DNP, the partial restoration of the ionomycin response in the presence of 1 mM ATP and 0.5 mM DNP can be ascribed to a release of Ca<sup>2+</sup> from intracellular stores via some mechanism which is dependent on ATP. The data suggest that there is an interaction between ATP and ionomycin which increases the intracellular Ca<sup>2+</sup> concentration not via mitochondrial activity but via some other mechanism which requires both ATP and ionomycin. As the oocytes had to be preincubated with the mitochondrial inhibitors for them to be effective, it is likely that the ATP dependent process which is necessary for release of Ca<sup>2+</sup> to occur is an ATP-dependent loading of the calcium store with Ca<sup>2+</sup>. As mentioned previously the endoplasmic reticulum contains Ca<sup>2+</sup> ATPases, these ATPases mediate the process of Ca<sup>2+</sup> uptake.

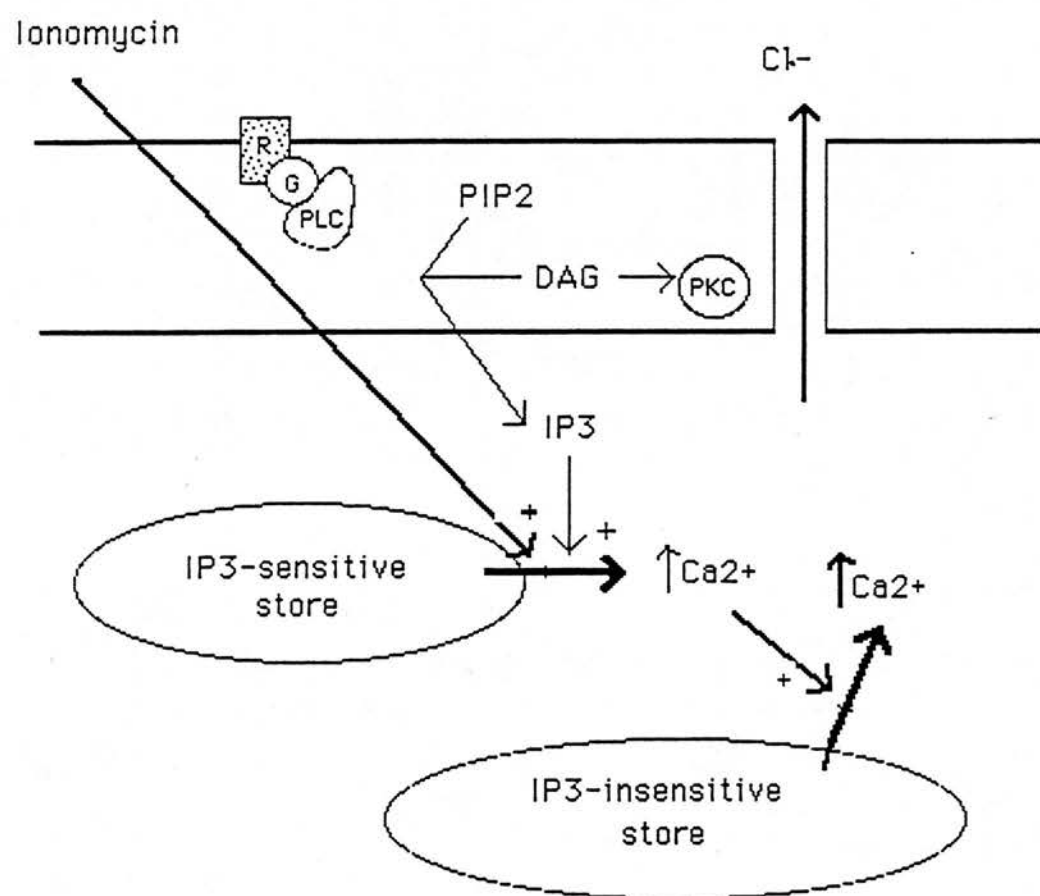
It has been suggested for a number of cell types that there are two distinct intracellular Ca<sup>2+</sup> stores. For example in smooth muscle cells (Heasley &

Rahwan, 1982), two types of sarcoplasmic reticulum (SR) have been described in according to its location; superficial ST located close to the inner surface of the sarcolemma and deep SR lying in the interior. Indeed, a two compartment model has been proposed for smooth muscle SR (van Breemen & Saida, 1989). The SR contains two separate types of  $\text{Ca}^{2+}$  release channels, i.e. a channel insensitive to PTX but sensitive to  $\text{Ca}^{2+}$ , ATP, and caffeine and a channel activated by  $\text{IP}_3$ . They have shown that  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release raises the intracellular concentration of  $\text{Ca}^{2+}$ , resulting in activation of caffeine-sensitive  $\text{Ca}^{2+}$  release channels (van Breemen *et al.*, 1988; van Breemen & Saida, 1989), by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. 97% of SR  $\text{Ca}^{2+}$  is releasable by  $\text{IP}_3$  (Yamamoto & van Breemen, 1985).  $\text{IP}_3$ , generated through agonist-induced activation of receptors, opens  $\text{Ca}^{2+}$  channels in the superficial SR, resulting in the fast component of the response.  $\text{IP}_3$ , however, is hydrolized by its specific phosphatase before it reaches the deep SR, but a flow of  $\text{Ca}^{2+}$  from the superficial to the deep SR induces a  $\text{Ca}^{2+}$ -induced slow  $\text{Ca}^{2+}$  release, resulting in a slow phase of the response. The *Xenopus* oocyte does not contain SR, but a similar system may operate.

In conclusion, the ionomycin response, consisting of fast and slow components, can be a model of responses observed in expressed receptors in *Xenopus* oocytes. Ionomycin directly releases  $\text{Ca}^{2+}$  from the same intracellular stores as  $\text{IP}_3$ , whose function is dependent on the mitochondrial activities, resulting in the activation of the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. With the use of ionomycin it has been confirmed that there are two distinct calcium stores, one of which gives rise to a fast transient inward current, the other which produces a slow inward current response. The fast response is more dependent on  $\text{Ca}^{2+}_i$  and mitochondrial inhibitors. The slow component depends on  $\text{Ca}^{2+}$  entry from the extracellular environment. A cell model of the events which may occur during the response to ionomycin (or upon receptor activation) are shown in Figure 7.9.

**Figure 7.9** A cell model of the response to ionomycin.

This diagram shows a cross-section through the cell membrane of a *Xenopus* oocyte and outlines the events which occur between the application of a receptor agonist or ionomycin and the production of a chloride current response. It indicates that ionomycin causes the release of  $\text{Ca}^{2+}$  from an  $\text{IP}_3$ -sensitive store.



## SECTION 8

### The endogenous response to growth hormone-releasing hormone (GRH)

In *Xenopus* oocytes at stages V and VI (Dumont, 1972), which have intact follicle cell layers, slow membrane current responses have been reported to a number of hormones and neurotransmitters. These responses are thought to reside in the follicle cells, the macrovilli of follicle cells contact with microvilli of the oocyte via gap junctions (Browne, Wiley & Dumont, 1978) and hence the response is conducted to the oocyte and is measurable by the two-electrode voltage clamp method.

The first endogenous receptor responses to be reported were those to catecholamines and adenosine (Kusano, Miledi & Stinnakre, 1977,82; Lotan, Dascal, Cohen & Lass, 1982) where the response was associated with an increase in membrane conductance and carried by  $K^+$ . Thus far, further reported responses include those to gonadotrophins, vasoactive intestinal peptide (VIP)(Woodward & Miledi, 1987 a, b), oxytocin, prostaglandins (Miledi & Woodward, 1989), corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP)(Moriarty, Gillo, Sealfon & Landau, 1988). The responses to these various neurotransmitters/ hormones appears to be mediated by distinct receptors which are coupled to an endogenous intracellular messenger pathway involving cyclic nucleotides. The different receptors are all thought to stimulate adenylate cyclase, causing an increase in intracellular adenosine 3'5'-cyclic monophosphate (cAMP) which in turn, probably through activation of cAMP-dependent protein kinases regulates the gating of specific  $K^+$  channels (Lotan, Dascal, Oron, Cohen & Lass, 1985; Van Renterghem, Penit-Soria & Stinnakre, 1985; Stinnakre & Van Renterghem, 1986; Smith, Brooker & Brooker, 1987; Woodward & Miledi, 1987 a, b).

The following section reports the existence of an endogenous receptor in *Xenopus* oocytes for growth hormone-releasing hormone (GRH), which upon the application of GRH (1nM-1 $\mu$ M) induces a slow membrane current response. A species specificity was observed among GRHs derived from different animals (rat, cow, pig, human). This finding is discussed in connection with the interaction between GRH molecules and the GRH receptor.



## Establishment that the GRH response is dependent on the activation of GRH receptors

### Species specificity of the GRH response

The effect of growth hormone-releasing hormone on follicular *Xenopus* oocytes was tested. Figure 8.1 A shows whole-cell current responses induced in follicular *Xenopus* oocytes, at a holding potential of -60 mV, upon the application of GRHs (0.1  $\mu$ M) of four different species (rat, bovine, porcine and human). The only peptide which elicited a response was rat GRH which produced a smooth outward current response of 70nA in a *Xenopus laevis* oocyte which was uninjected and had an intact follicle cell layer (i.e. follicular oocytes). Figure 8.1B shows the effect of the addition of four different species (rat, bovine, porcine and human) of growth hormone releasing hormone (GRH) to *Xenopus* oocytes which have had their follicular cell layer removed by treatment with collagenase (1mg/ml). In these oocytes the application of GRH has no effect on the membrane current.

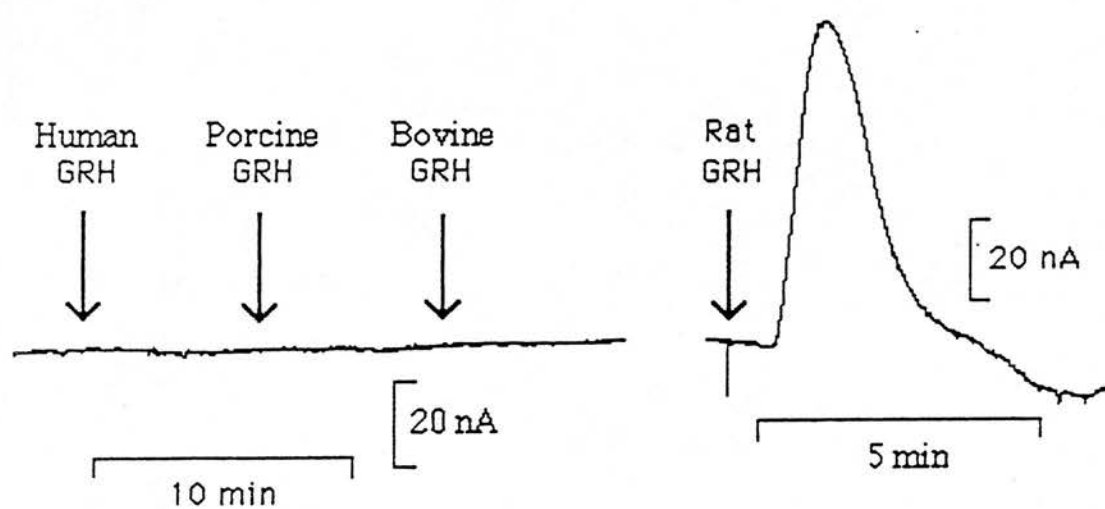
It is important to note that only rat GRH 1-29, consisting of the initial 29 amino acids of a peptide which has a full length of 42 amino acids (Speiss, Rivier & Vale, 1983), was effective in inducing a response of a transient outward current ( $n=5$ ). Other GRHs (bovine, porcine and human) shown in Fig. 1 (A & B) were complete forms consisting of 44 amino acids. From this finding the possibility that the *Xenopus* oocyte is responding to a short peptide sequence arises. However, a fragment of 1-29 bovine GRH also failed to evoke any significant changes in the membrane current. In addition, the complete form of rat GRH 1-42 was available later in the present study, and the application of this complete form also induced a transient outward current which is shown in Fig. 8.2. This oocyte gave rise to a transient outward current of 60nA to rat GRH (1-29) (0.1 $\mu$ M). Following washing with standard solution for 30 min, the oocyte was exposed to rat GRH (1-42) (1 $\mu$ M) and produced an outward current of 30nA (the amplitude of the second current response may be reduced due to desensitisation of the receptors). Therefore a likely explanation for the *Xenopus* oocyte responding to only rat GRH is because of its amino acid sequence. Such a species specificity of the GRH response in *Xenopus* oocytes can possibly be ascribed to the prominent difference in the structure of the rat GRH from that of bovine, porcine and human GRH. Such a species specificity may suggest that the *Xenopus* GRH has a greater sequence homology with rat GRH than with bovine, porcine or human. Unfortunately, however, frog GRH



Figure 8.1. Effect of growth hormone-releasing hormone (GRH) on follicular and denuded oocytes of *Xenopus laevis*.

Four distinct GRHs of different species origin (rat, bovine, porcine, human) were tested to examine the species specificity of the GRH response. Whole-cell currents were recorded from oocytes using the two-electrode voltage-clamp method. The holding potential was -60 mV for all cases. (A) Current traces obtained from a follicular oocyte. A transient outward current was induced only by rat GRH. (B) Records obtained from a denuded oocyte. No significant changes in the current was induced by any GRHs of four species origin. The point of application of GRH or washout with standard solution is indicated by an arrow. GRH was introduced to and removed from the experimental chamber by perfusion. Current and time calibrations are the same for both traces. Outward and inward currents are shown as upward and downward deflections respectively in all figures.

A



B

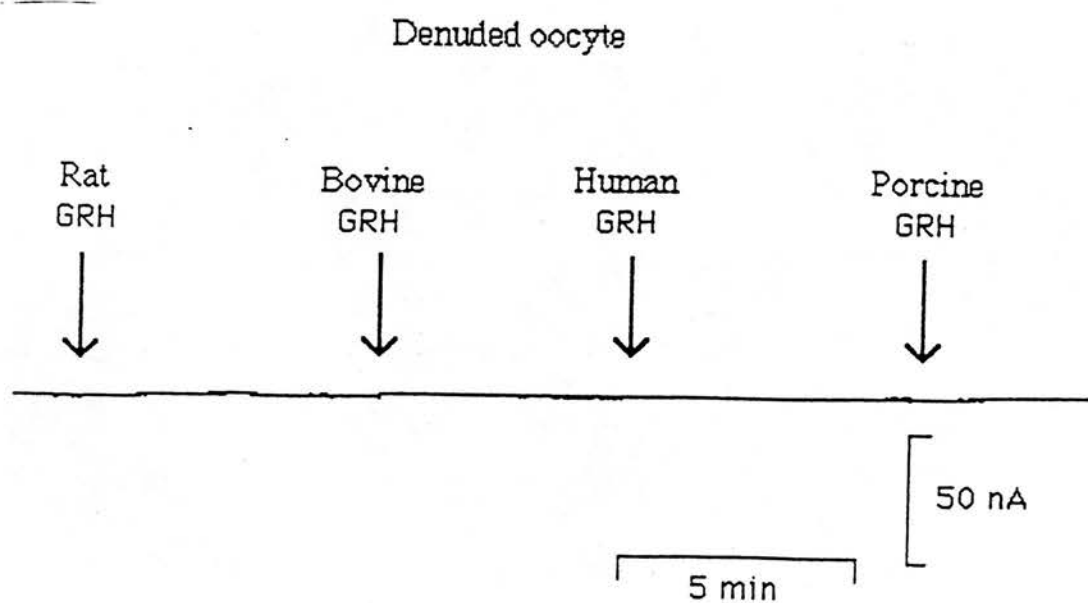
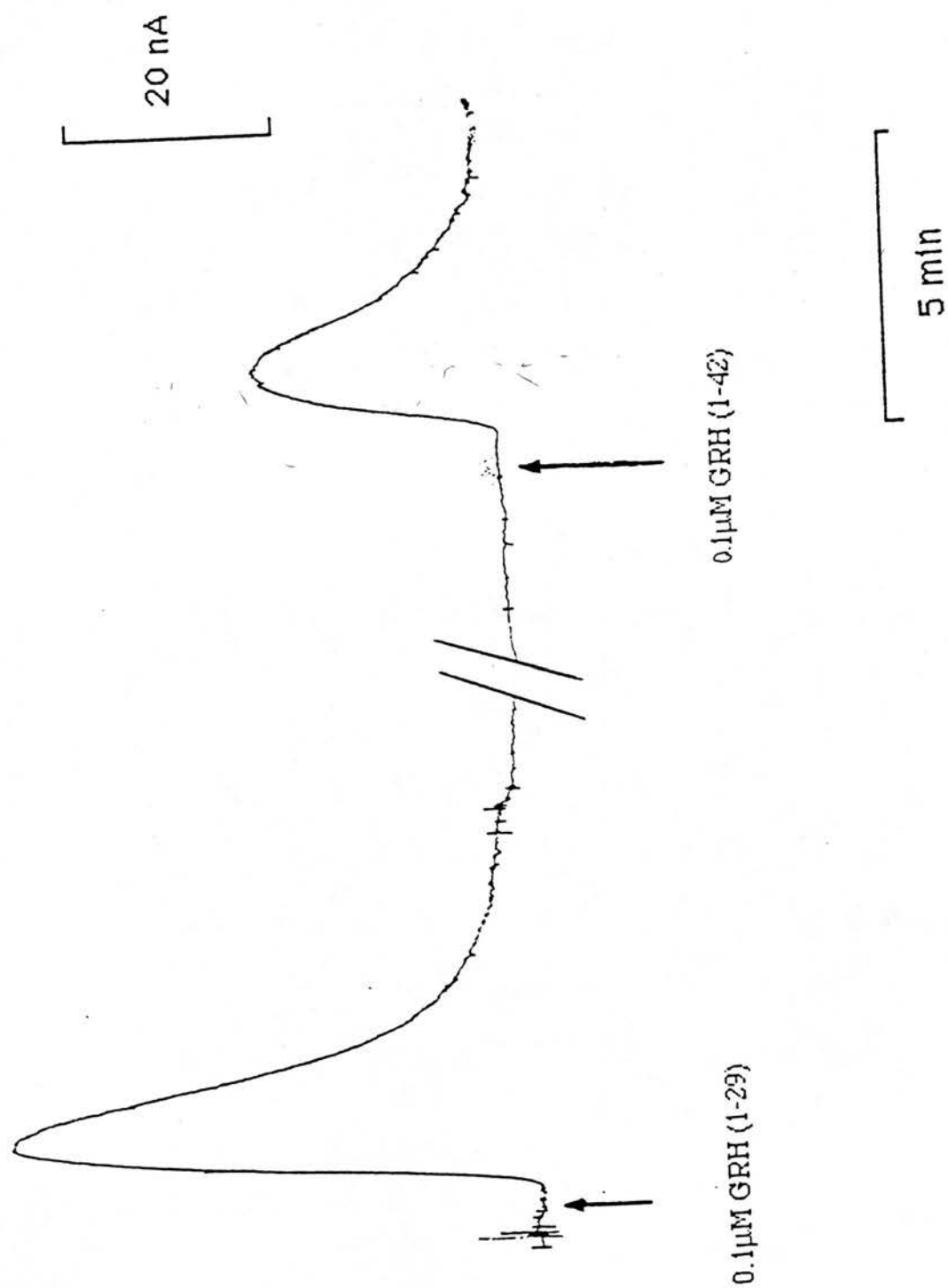


Figure 8.2. Effect of different lengths of rat GRH peptide.

The current responses elicited by the application of the first 29 amino acids of rat GRH (GRH 1-29) and the full length rat GRH (GRH 1-44) both at a concentration of  $0.1\mu\text{M}$  in a *Xenopus laevis* oocyte. The interruption in the trace is for washing with standard solution for 30 minutes.



is not available to study this possibility. These differences in the structures of the GRH types used in the present study are detailed in the discussion section.

### **The effect of known endogenous receptor blockers.**

As mentioned previously, a number of endogenous responses have been shown to exist in the follicular cell layer of *Xenopus* oocytes, all of which have a similar response profile to the GRH response. That the response to GRH can only be elicited by a peptide with a specific sequence suggests that a specific receptor is involved in the response seen to GRH. To confirm that the response to GRH was not due to the activation of one of the endogenous receptors already characterised, the effect of several known endogenous receptor blockers was tested on the GRH response.

The response to GRH (0.1  $\mu$ M) was not affected by the acetylcholine blocker atropine (0.1  $\mu$ M) ( $n=3$ ), which has been shown to inhibit the ACh response in *Xenopus* oocytes (Kusano, Miledi & Stinnakre, 1982). An example of the response to GRH (0.1 $\mu$ M) in the presence of atropine (0.1 $\mu$ M) is shown in Figure 8.3 A. Therefore the response produced by GRH could not be due to the activation of endogenous ACh receptors. The GRH response was not affected by the  $\beta$ -adrenergic blocker timolol (2 $\mu$ M) or the purinergic (P1) blocker theophylline (0.1mM) as shown in figure 8.3 B and hence the GRH response was not due to the activation of these endogenous receptors. Specific antagonists to gonadotrophins or VIP were not available.

### **Characterisation of the GRH response.**

#### **Membrane currents elicited by GRH**

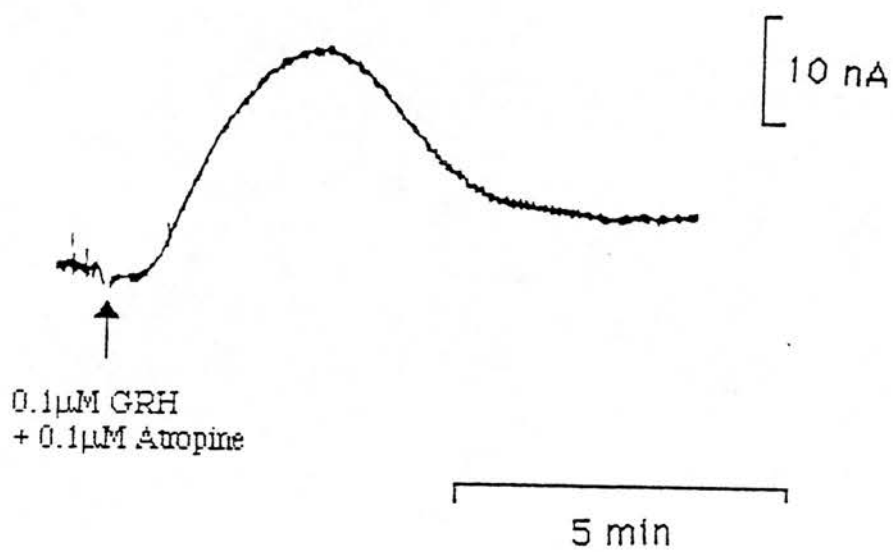
Rat GRH was applied to 90 oocytes from 18 frogs and 60% responded with an outward current response. An example of such an oocyte response is shown in Figure 8.4 A where the application of 0.1  $\mu$ M GRH induced a smooth outward current response of 70nA. In this oocyte continuous exposure to GRH resulted in a decay in the current, suggesting desensitization of GRH receptors and a further application of GRH would not induce a response unless the oocyte had been washed with standard solution for at least 20 min. As is the case with other endogenous responses from *Xenopus* oocytes the sensitivity to GRH varied considerably from frog to frog. In the most responsive oocytes the application of GRH (0.1 $\mu$ M) could elicit responses of 409 nA, whilst in others no response could be elicited, even with 1 $\mu$ M.

Figure 8.3 The effect of known endogenous receptor blockers on the response rat GRH.

(A) The response elicited in response to GRH ( $0.1\mu\text{M}$ ) in the presence of the acetylcholine blocker atropine ( $0.1\mu\text{M}$ ).

(B) The GRH response ( $0.1\mu\text{M}$ ) was not affected by the  $\beta$ -adrenergic blocker timolol ( $2\mu\text{M}$ ) or the purinergic blocker theophylline ( $0.1\text{mM}$ ).

A



B

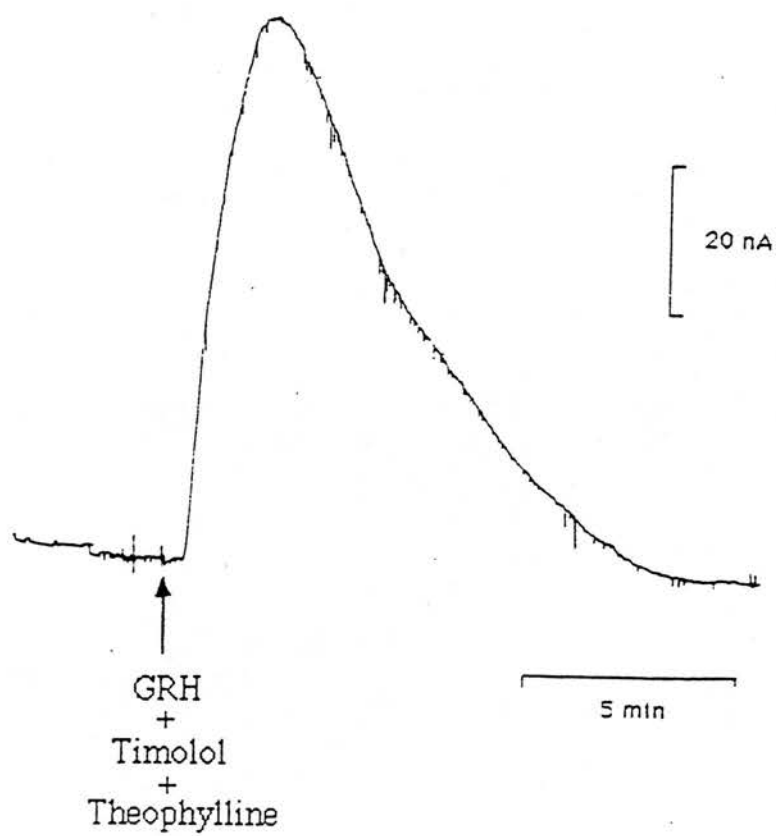
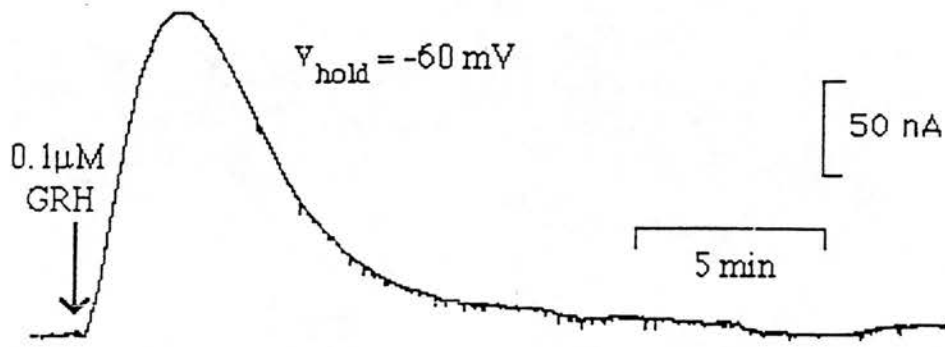


Figure 8.4 Characteristics of the response to GRH.

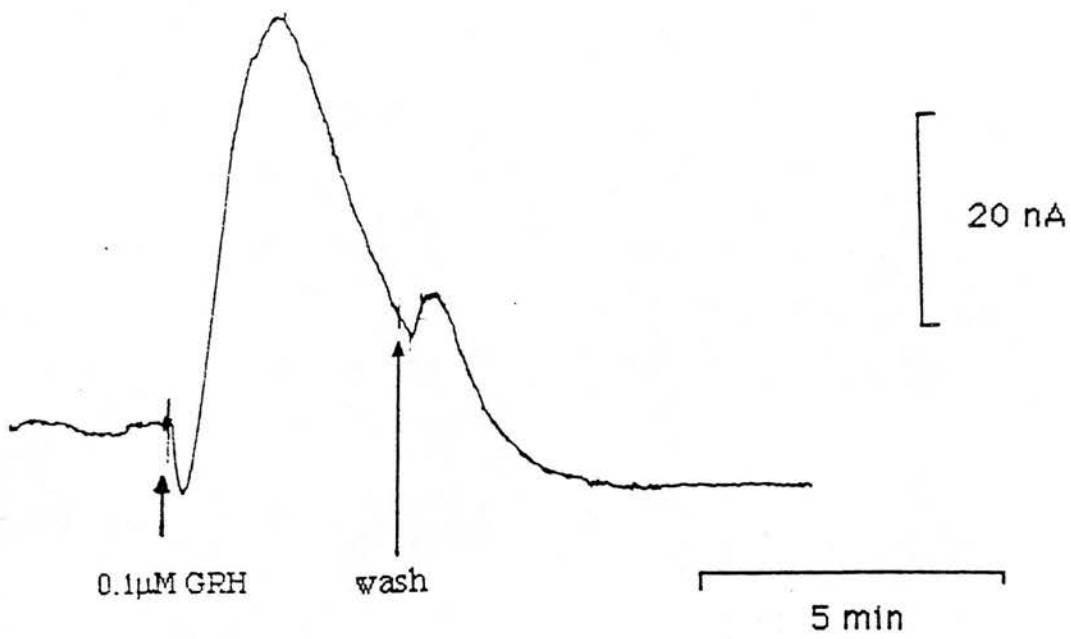
(A) shows a typical response to GRH ( $0.1\mu\text{M}$ ) in a follicular oocyte clamped at  $-60\text{ mV}$ . The upward deflection of the current trace indicates an outward current. In (B) an oocyte which shows an initial inward current response prior to the outward current elicited by GRH ( $0.1\mu\text{M}$ ).



A



B



Usually, the GRH response consisted of a transient outward current as mentioned above. Some oocytes, however, showed a fast transient inward current which was small in amplitude (less than 10 nA at 0.1  $\mu$ M GRH). A representative example of such a fast transient current response to GRH is shown in Figure 8.4 B, where the application of 0.1  $\mu$ M GRH induced a fast inward current response of 7 nA followed by a slow outward current of 44 nA. A similar inward current component has been reported in ACh responses from *Xenopus* oocytes (Lotan, Dascal, Cohen & Lass, 1982; Dascal, Landau & Lass, 1984) and is thought to be due to the activation of a  $\text{Cl}^-$  current.

The membrane conductance changed during the GRH response, as monitored by constant voltage pulses (Fig. 8.5). The GRH response was slow in time course and the change in the conductance occurred gradually. The conductance at the peak of the response to 0.1  $\mu$ M rat GRH was approximately twice the resting conductance ( $n=3$ ). In the oocyte shown in Figure 8.5, the resting conductance was 2.5  $\mu$ S. At the peak of the response the conductance of the membrane increased to 5  $\mu$ S. Therefore, the calculated conductance responsible for the GRH response, obtained by subtracting the resting conductance from the peak conductance, was 2.5  $\mu$ S.

### Dose-dependence of the GRH response

The GRH response occurred in a dose-dependent manner within a single oocyte, as shown in Figure 8.6. In this oocyte, washing with standard solution for longer than 20 min after each application of GRH was necessary to exclude desensitization of receptors. The holding potential was -60 mV in all cases. When the concentration of GRH was increased, the amplitude of the response became larger and the latency became shorter. The values of the amplitude (open circles) and potassium conductance ( $g_K$ ; filled circles) at different concentrations of GRH are plotted in Fig. 8.7 A. The latency, time to peak and half decay-time are plotted in Figure 8.7B.

### Ionic basis of the GRH response

As the GRH response consisted of outward current at a holding potential of -60 mV, it seemed likely that the current was due to an efflux of  $\text{K}^+$ , as has been found for other endogenous receptors. To test this hypothesis, the effect of the  $\text{K}^+$  channel blocker tetraethylammonium ( $\text{TEA}^+$ ) was examined on the GRH response ( $n=5$ ). As shown in Figure 8.8, the response to GRH induced the

Figure 8.5 Conductance change seen during the response to rat GRH.  
Voltage pulses of constant amplitude (1 sec duration, 0.5 Hz) were applied to the oocyte to monitor changes in the conductance. The upper trace shows the membrane current and the lower trace shows the holding potential.

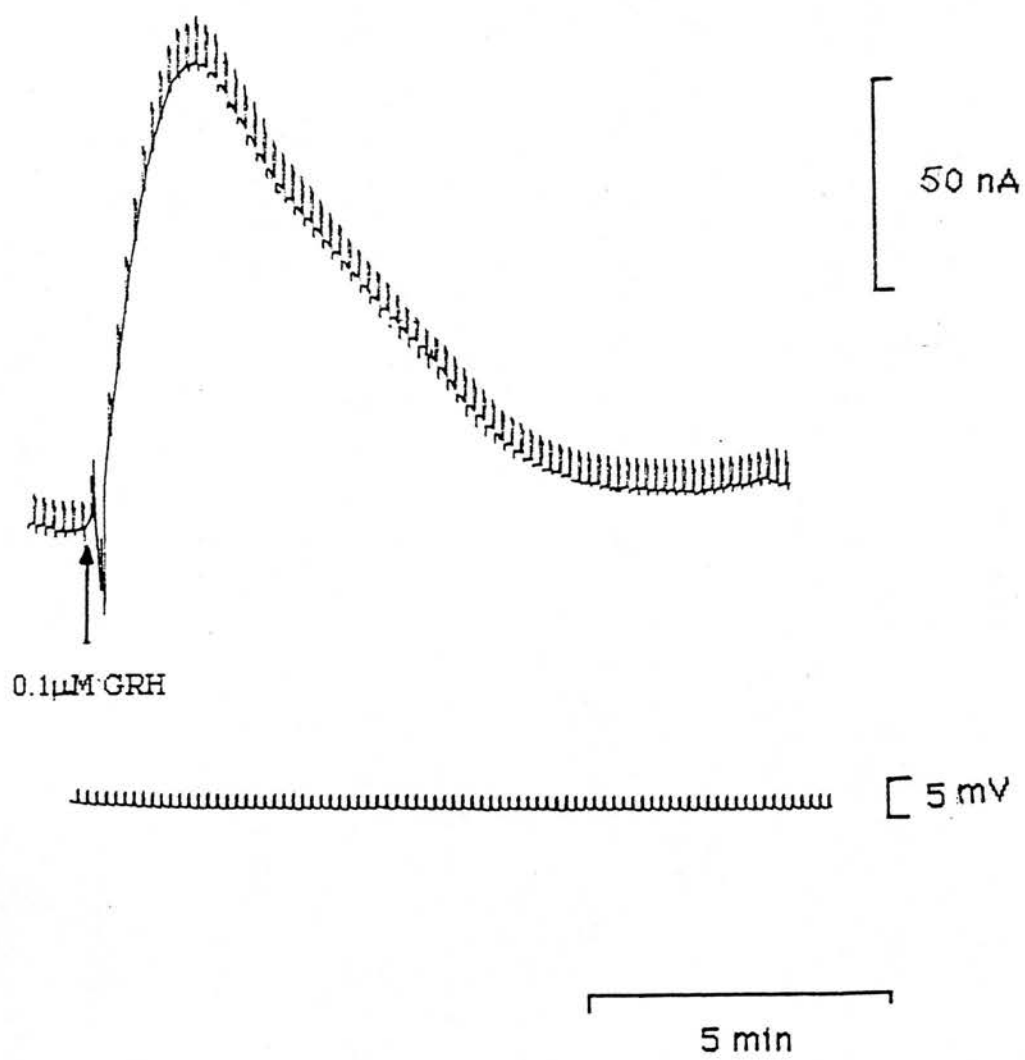


Figure 8.6. Dose-dependence of current responses elicited by rat GRH in a follicular *Xenopus* oocyte. Different concentrations of GRH was applied to the same oocyte, and it was washed with standard solution for approximately 30 min for each trial with GRH. The concentration of GRH is indicated below each current trace. Voltage pulses of constant amplitude (1 sec duration, 0.5 Hz) were applied to the oocyte to monitor changes in the membrane conductance. Thicker line indicates an increase in the conductance. The point of application of GRH is indicated by an arrow. Current and time calibrations apply to all traces.

200 nA  
5 min

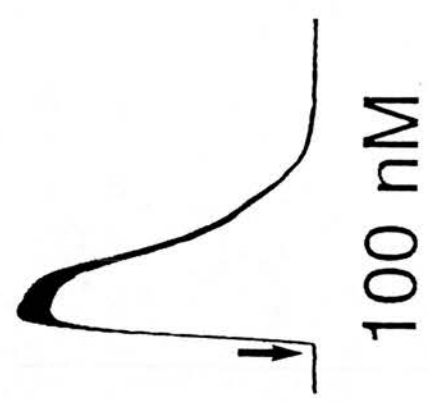


Figure 8.7. Dose-dependence of parameters of responses against the concentration of rat GRH. The concentration is in logarithmic scale. A: Normalized values of the amplitude (open circles with the solid line) and the conductance (filled triangles with the dashed line) of the GRH responses. B: Plots of latency (open squares with the solid line), time-to-peak (open circles with the solid line) and half-decay time (filled triangles with the dashed line). The data were obtained from the oocyte shown in Fig. 8.6.

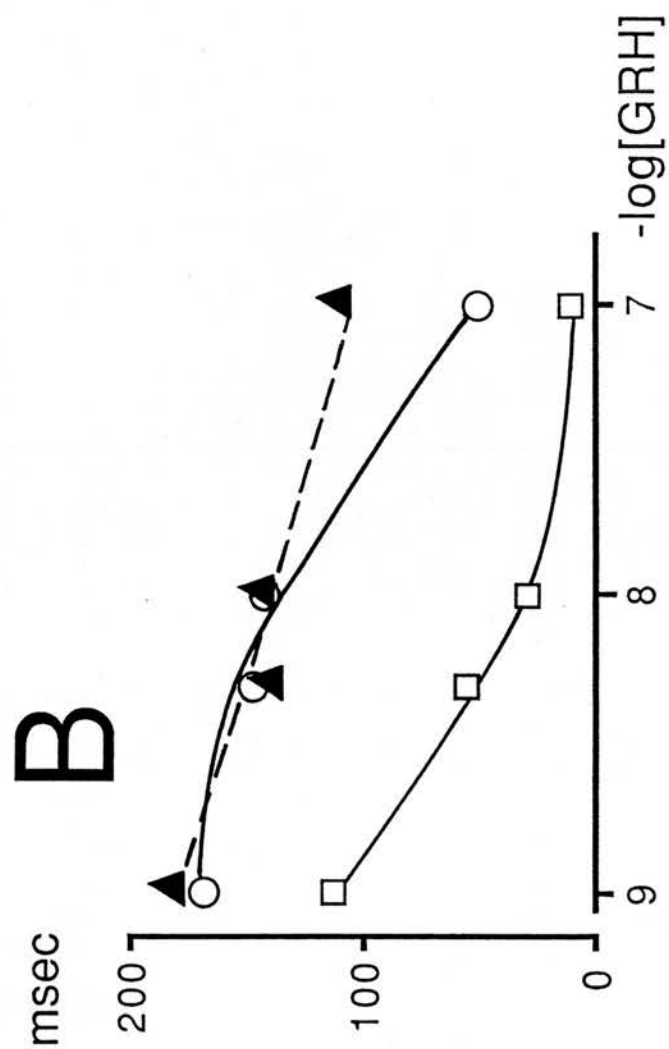
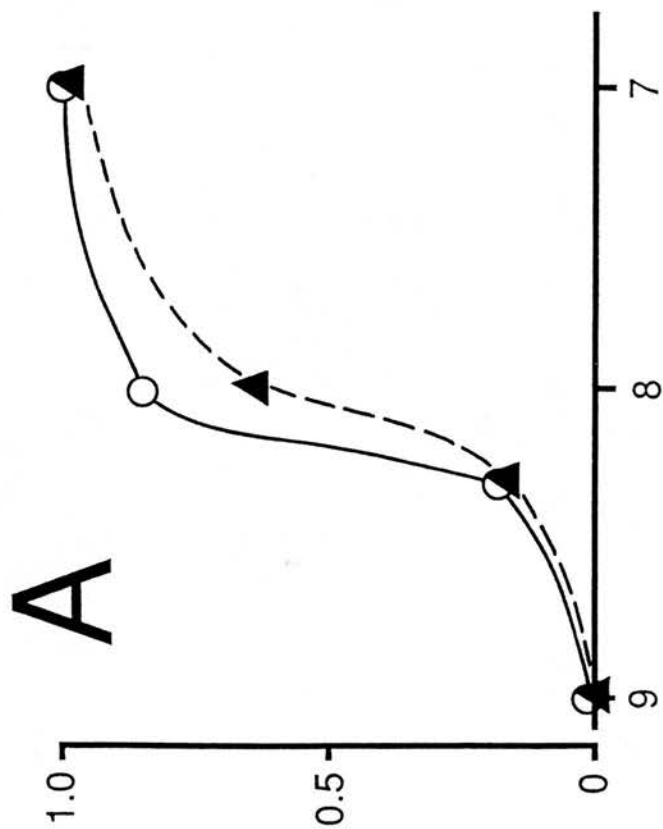
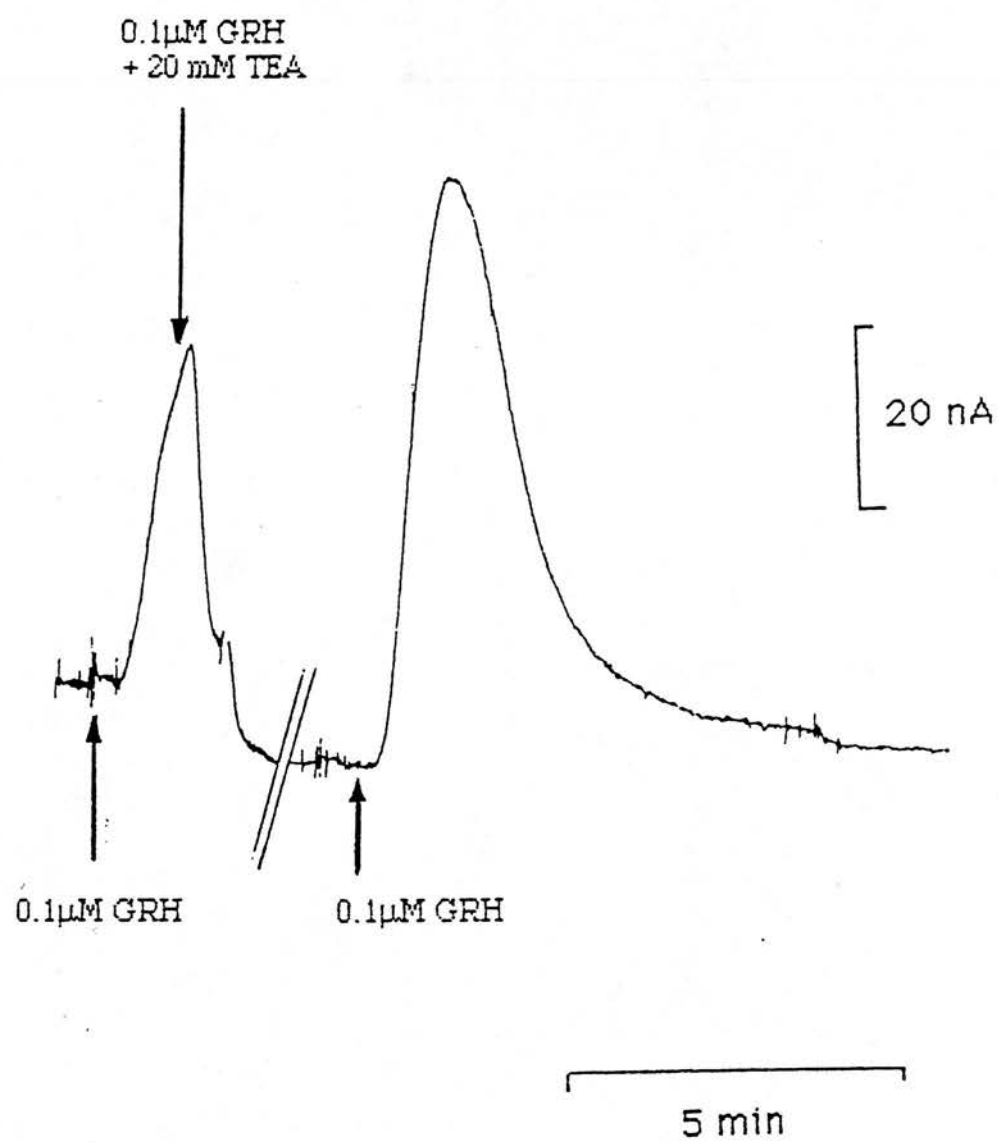




Figure 8.8 The inhibition of the GRH response by the  $K^+$  channel blocker TEA<sup>+</sup>

The application of GRH (0.1 $\mu$ M) elicits an outward current which is abolished by the subsequent application of TEA<sup>+</sup> (20 mM) in the continued presence of the GRH. The interruption in the trace is for the washing of the oocyte with standard solution for 30 mins. A subsequent application of GRH (0.1 $\mu$ M) to the same oocyte elicits an outward current.



expected outward current. Before this current reached a peak, TEA<sup>+</sup> (20mM) in the continued presence of 0.1 $\mu$ M GRH truncated the response to GRH (to 35nA). That TEA<sup>+</sup> blocked the response to GRH is shown by a subsequent application of GRH (0.1 $\mu$ M) after 30 minutes of washing the oocyte with standard solution. In the absence of TEA<sup>+</sup> the response to GRH was larger, at 60 nA.

Further evidence for the involvement of K<sup>+</sup> in the response to GRH was obtained by measuring the reversal potential of the outward current. The reversal potential measurement was carried out in two ways; firstly, the ramp method (see methods) which involved repetitively applying voltage ramps of 0.8 - 1.0 sec duration with slopes of 80 - 120 mV/sec. It is assumed that the total membrane current  $I_m$ , which is evoked by a ramp-voltage during the GRH response, is given by summation of current carried by K<sup>+</sup> ( $I_K$ ) and currents carried by ions other than K<sup>+</sup>, leakage currents ( $I_l$ ). Thus,  $I_m = I_K + I_l = g_K(V - E_K) + \sum g_l(V - E_l)$ , where  $V$  is the membrane potential and  $E_K$  and  $E_l$  indicate the reversal potential for K<sup>+</sup> and for ions other than K<sup>+</sup>, respectively. According to this equation, all current traces evoked by repetitive voltage-ramps during the GRH response must meet at the same point (i.e. at  $E_K$ ), where  $I_m$  takes the constant value  $\sum g_l(E_K - E_l)$  because the driving force for K<sup>+</sup>, i.e. ( $V - E_K$ ), becomes zero. Indeed, this was the case as shown in Fig. 8.9 A, where a family of membrane currents evoked by repetitive voltage ramps during the GRH response meet at the same point. The oocyte was bathed in standard solution whose external K<sup>+</sup> concentration was 2 mM. The reversal potential for the GRH response was measured from the membrane potential on the voltage-ramp trace where all current traces crossed, and the value was -105 mV. The mean reversal potential obtained from 4 oocytes by this ramp method was  $-101.3 \pm 3.6$  mV. Again, the reversal potential for the GRH response was around -100 mV. The reversal potential of approximately -100 mV estimated by the two methods is compatible with the reported reversal potential for K<sup>+</sup> in *Xenopus* oocytes (Barish, 1983; Lotan, Dascal, Cohen & Lass, 1982; Kusano, Miledi & Stinnakre, 1982).

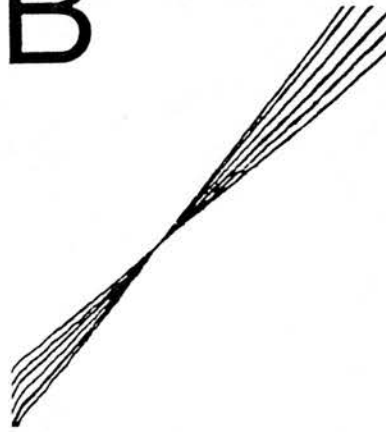
Dependence of the GRH response on K<sup>+</sup> was further assured by changing the external concentration of K<sup>+</sup>. The bathing medium was switched from normal 2 mM to 10 mM for the same oocyte shown in Fig. 8.9 B, and another family of membrane currents were obtained by the repetitive ramp method during the GRH response. In this oocyte the reversal potential for the GRH response, measured from the meeting point, was -63 mV. The depolarizing shift in the reversal potential, therefore, was 42 mV in this oocyte when external K<sup>+</sup>

Figure 8.9 The reversal potential of the response to GRH and measurement of a shift in the reversal potential for the GRH response by varying external concentration of  $K^+$ . A: A family of membrane currents (upper column) obtained by applying repetitive voltage ramps (lower column) to an oocyte during a response to  $0.1 \mu M$  rat GRH. The oocyte was bathed in normal solution ( $2 \text{ mM } K^+$ ). Although ramps were applied at  $0.5 \text{ Hz}$ , the number of current traces is reduced for illustration. B: A family of currents recorded from the same oocyte in high- $K^+$  ( $10 \text{ mM}$ ) solution. Current, voltage and time scales are the same for both records.

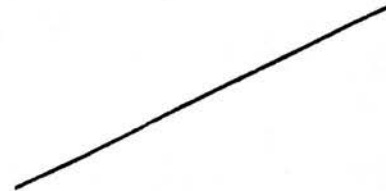
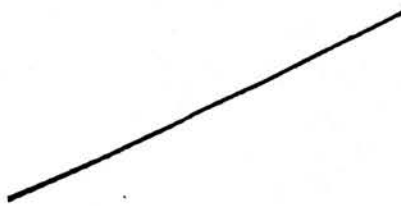
A



B



200 nA



0.5 sec

0  
-50  
-100  
(mV)

concentration was changed from 2 to 10 mM. The mean reversal potential for the GRH response in high  $K^+$  solution was  $-61.3 \pm 2.9$  mV ( $n=4$ ). The average depolarizing shift in the reversal potential for the GRH response, therefore, was 40 mV. This value of 40 mV is in a very good agreement with the expected value of 40.7 mV calculated from the Nernst equation at 22°C. This agreement indicates that the GRH response is exclusively dependent on  $K^+$ .

In contrast, the reversal potential for the GRH response did not significantly change when the external  $Cl^-$  concentration was decreased to a third of that of standard solution by substitution with membrane-impermeant methanesulphonate $^-$  (Sharp & Thomas, 1981). Under such conditions the mean value for the reversal potential was  $-100.7 \pm 3.2$  mV ( $n=3$ ). The GRH response also maintained a reversal potential of around -100 mV ( $-101.3 \pm 1.7$  mV;  $n=3$ ) when the external  $Na^+$  concentration was decreased to a fifth of that of standard solution by substituting  $Na^+$  with Tris (See Methods). These results show that the GRH response is predominantly dependent on  $K^+$  but not on  $Na^+$  or  $Cl^-$ .

### **Dependence of the GRH response on $Ca^{2+}$**

The effect of the internal concentration of  $Ca^{2+}$  on the GRH response was studied using BAPTA/AM. BAPTA/AM is a chelator of internal  $Ca^{2+}$ ; it penetrates the cell membrane and is cleaved by cytoplasmic esterases to yield free BAPTA which remains trapped in the cell (Tsein, 1981) where it binds free  $Ca^{2+}$ . Oocytes were treated with 10  $\mu$ M BAPTA/AM (external application) for 2-3 h, which was sufficient to chelate internal  $Ca^{2+}$  released from stores, eg. inhibition of the activation of  $Ca^{2+}$ -dependent  $Cl^-$  channels by ionomycin (see section 7). The GRH response was not affected by BAPTA/AM treatment (10 $\mu$ M for 3 hours)( $n=4$ ) as shown in Fig.8.10 where the application of GRH (0.1  $\mu$ M) produces the usual outward current response of 30nA.

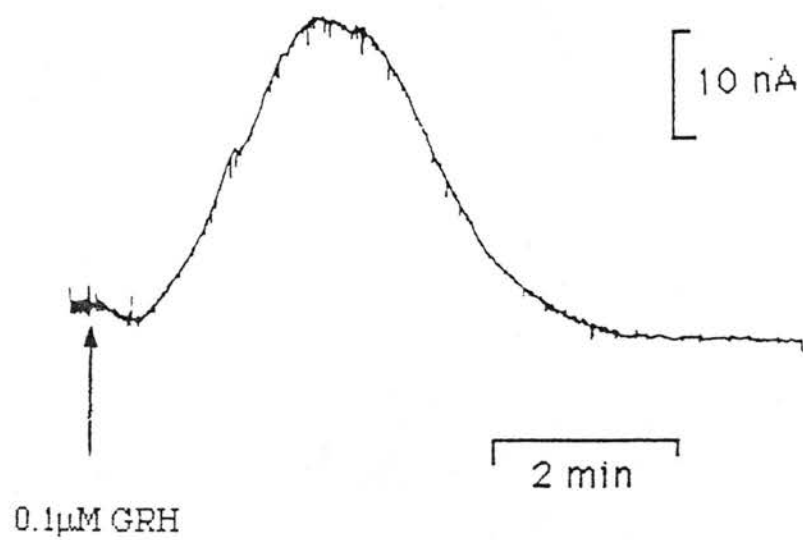
### **Effect of forskolin on the GRH response**

Cyclic AMP is implicated in mediating the  $K^+$  currents elicited by  $\beta$ -adrenergic agonists, adenosine, gonadotropin and VIP (Lotan, Dascal, Oron *et al.*, 1985; Van Renterghem, Penit-Soria & Stinnakre, 1985; Woodward & Miledi, 1987a,b). The  $K^+$  current elicited by the activation of these endogenous receptors and GRH of the present study resembles that induced by adenylate cyclase activator forskolin or by intra-oocyte injection of cyclic AMP

Figure 8.10 The effect of the calcium chelator BAPTA/AM on the GRH response.

The application of GRH ( $0.1\mu\text{M}$ ) elicits an outward current in an oocyte which has been pretreated with BAPTA/AM ( $10\mu\text{M}$ ) for 3 hours.

Pre-incubated with 10  $\mu$ M BAPTA/AM





(Woodward & Miledi, 1989). As the GRH response was not dependent on internal  $\text{Ca}^{2+}$ , the possibility that the response may be dependent on the cyclic AMP pathway was tested. It has been shown that at low concentrations, which are unable to elicit a potassium current, forskolin potentiates oocyte responses to isoprenaline and adenosine (Van Renterghem, Penit-Soria & Stinnakre, 1985). In the present study  $0.4\mu\text{M}$  forskolin did not induce any current, but did affect the response to GRH, as shown in Figure 8.11. A control GRH response was evoked by  $0.1\mu\text{M}$  rat GRH(1-29); the amplitude of the response was 68 nA. The oocyte was washed with standard solution for 50 min. Following this the oocyte was perfused with standard solution containing  $0.4\mu\text{M}$  forskolin for 5 min followed by the application of  $0.1\mu\text{M}$  rat GRH(1-29) in the presence of  $0.4\mu\text{M}$  forskolin. The second GRH response was potentiated by forskolin; its amplitude was 98 nA. The amplitude of the GRH response in the presence of forskolin ( $0.4\mu\text{M}$ ) was increased by  $170.1 \pm 25.4\%$  (mean  $\pm$  S.D;  $n=3$ ).

### **Distribution of $\text{K}^+$ channels responsible for the GRH response**

Although rat GRH induced a transient outward current in follicular oocytes (Fig. 8.12 A), it could not evoke any significant response in mechanically or enzymically denuded oocytes ( $n=6$ ), as is shown in Fig. 8.12 B. The total membrane capacitance was measured in follicular and denuded oocytes; values were obtained from different oocytes with similar cell size (1.2-1.3 mm in diameter). The mean capacitance values were  $437 \pm 74$  nF and  $261 \pm 80$  for follicular and denuded oocytes, respectively ( $n=27$ ). The decrease in the total membrane capacitance indicates that the follicle cell layer was electrically connected to the oocyte membrane. This electrical coupling has been shown to be due to gap junctions between the oocyte and the follicular cell membrane (Brown, Wiley & Dumont, 1979; Browne & Werner, 1984; Van der Hoef, Dictus, Hage & Bluemink, 1984)). These gap-junctions are permeable to small ions and molecules including cAMP (Pitts & Simms, 1977, Lawrence, Beers & Gilula, 1978). Gap junctions can be inhibited by alkanols such as heptanol and octanol (Saez, Conner, Spray *et al.*, 1988; Paige-Lacy, McIntosh & McIntosh, 1989). To show that the response to GRH is dependent on the integrity of these gap-junctions the gap-junction inhibitor octanol was examined in intact follicular oocytes. After incubation in the presence of octanol (1mM) for 10-30 min, all oocytes showed a total membrane capacitance decrease from  $400.2 \pm 54$  nF to  $343.5 \pm 45$  nF, respectively ( $n=4$ ). In these octanol-treated oocytes, the sensitivity of the oocytes to GRH ( $0.1\mu\text{M}$ ) was reduced by 95%

Figure 8.11 Facilitatory effect of forskolin on the GRH response. A: A response to  $0.1\ \mu\text{M}$  rat GRH obtained from a *Xenopus* oocyte. B: The same oocyte shown in A was washed with standard solution for 30 min, pretreated with  $0.4\ \mu\text{M}$  forskolin for 5 min and was challenged by  $0.1\ \mu\text{M}$  rat GRH in the continuous presence of forskolin. The points of introduction of GRH into the experimental chamber are indicated by the arrows. Current and time calibrations apply to both records.

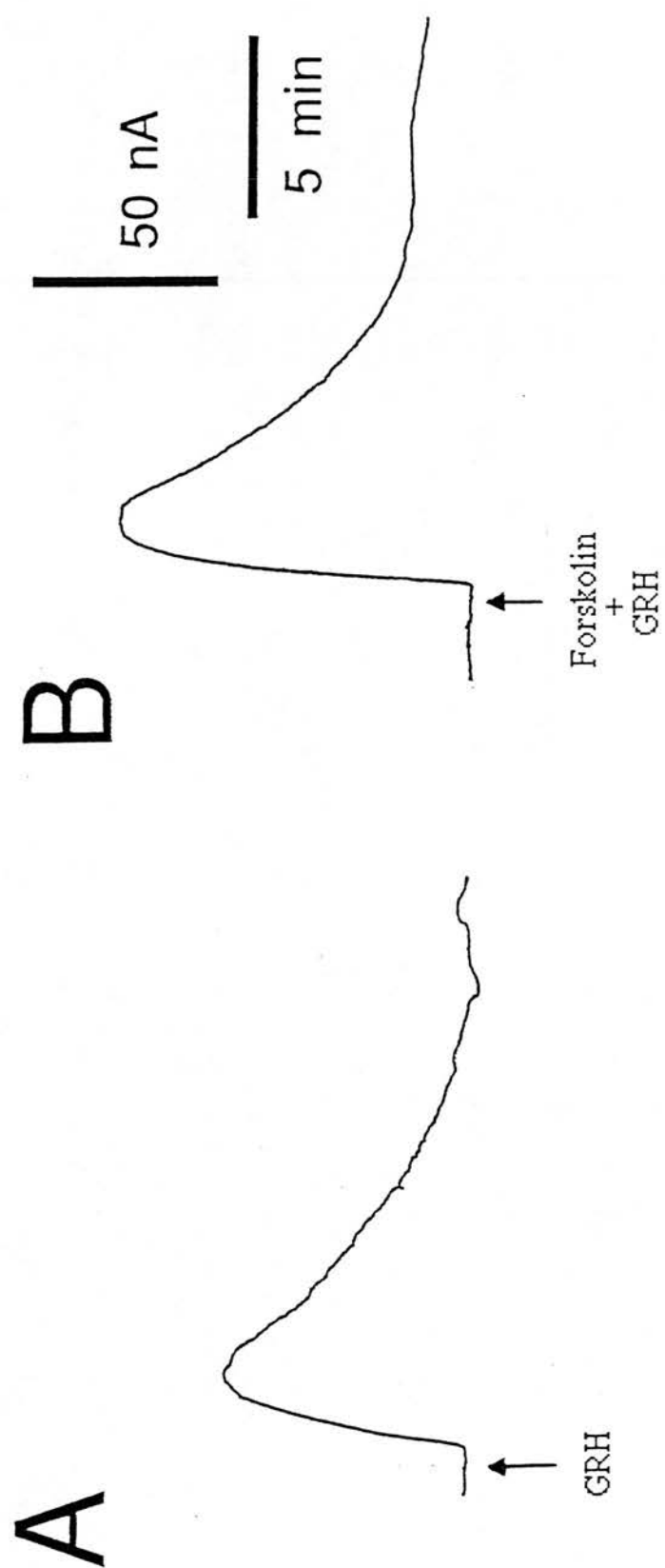
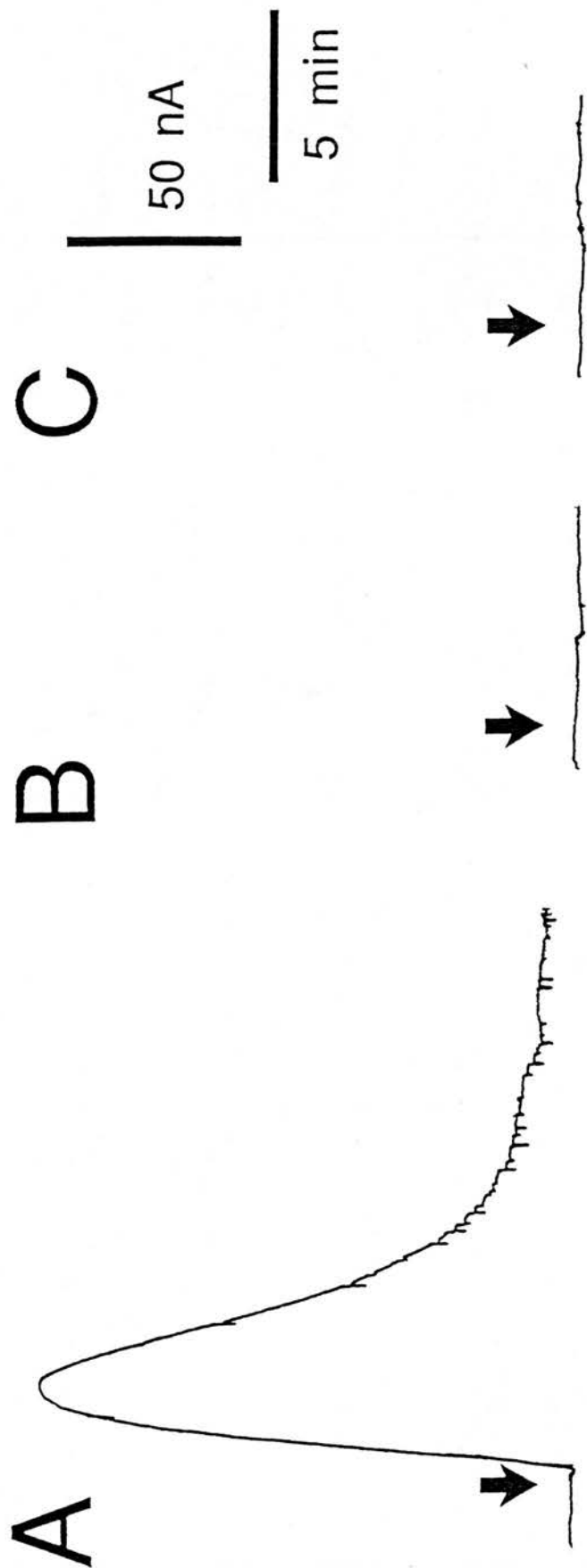


Figure 8.12. Disappearance of the GRH response by removing the connection between the oocyte membrane and the follicle cell layer. A: Control. A transient outward current was evoked by  $0.1\ \mu\text{M}$  rat GRH in an *Xenopus* oocyte with the intact follicle cell layer. B: An oocyte whose follicle cell layer was mechanically removed with forceps. C: An oocyte whose gap junctions, which electrically connect the oocyte and the follicle cell layer, were blocked by the application of  $1\ \text{mM}$  1-octanol for 11 min. Introduction of  $0.1\ \mu\text{M}$  rat GRH into the experimental chamber is indicated by an arrow in all records. Current and time scales are the same for all records.



(n=4). An example of an oocyte which had been treated with octanol is shown in Figure 8. 12 C, where the application of GRH failed to elicit a membrane response.

## DISCUSSION

Amino-acid sequences of GRHs from four different species (rat, bovine, porcine and human) used in the current study are shown in Figure 8.13 (Guillemin, Brazeau & Bohlen, 1982; Gubler, Monahan, Lomedico *et al.*, 1983; Spiess Rivier & Vale, 1983; Lance, Murphy, Sueir-Diaz & Coy, 1984; Mayo *et al.*, 1985a, 1985b; Frohman, Downs, Chomczynski & Frohman, 1989). It should be noted that the amino-acid sequence of rat GRH is quite different from those of bovine, porcine and human GRHs. Amino acids which are particular to the rat GRH are marked in bold face; only amino acids in the 1-29 region are marked because this region is important for function. For rat GRH, a fragment consisting of the initial 29 amino acids was used in the early experiments and its complete form of 43-amino-acid peptide became available later. For comparison, both the fragment consisting of the first 29-amino acids and the complete form of 44-amino-acid bovine GRH peptides were tested. GRHs from other two species (porcine and human) were complete forms consisting of 44 amino acids. In porcine and human GRHs, the amino acids in the 1-29 region are exactly the same, but some differences are observed in the later amino acids (not marked). Bovine GRH shows one different amino acid (marked in underlined bold face) in the 1-29 region in comparison with porcine and human GRHs; this **Asn** is also present in the same position in the rat GRH. Recently, the complete structure of mouse GRH consisting of 42 amino acids was deduced from its cDNA: His-Val-Asp-Ala-Ile-Phe-Thr-Thr-Asn-Tyr-Arg-Lys-Leu-Leu-Ser-Gln-Leu-Tyr-Ala-Arg-Lys-Val-Ile-Gln-Asp-Ile-Met-Asn-Lys-Gln-Gly-Glu-Arg-Ile-Gln-Glu-Arg-Ala-Arg-Leu-Ser (different amino acids from rat GRH are marked in bold face up to the 28th amino acid Asn) (Frohman, Downs, Chomczynski & Frohman, 1989). It is interesting that the amino-acid sequences of rat and mouse GRH are considerably different from each other although they are rodents. The amino acid sequence of mouse GRH is more similar to rat GRH than to other GRHs of higher mammals. Since it is suggested that the first amino acid is important for GRH molecules to bind to the GRH receptor (Frohman, Downs, Chomczynski & Frohman, 1989), it is probable that mouse GRH, whose first amino acid is also histidine as for rat GRH, may induce some response in follicle cells surrounding *Xenopus* oocytes. Unfortunately, however, this mouse GRH is not available at the

Figure 8.13 The amino acid sequences of four species of GRH. This figure shows the initial 29 amino acids of rat, bovine, porcine and human GRH peptides. The amino acids which are different from rat GRH are marked in bold face for the bovine, porcine and human GRH sequences.

Rat (1-43):

**His**-Ala-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Arg-Ile-Leu-Gly-  
Gln-Leu-Tyr-Ala-Arg-Lys-Leu-Leu-**His**-Glu-Ile-Met-Asn-Arg-  
Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Arg-Ser-Arg-Phe-Asn

Bovine (1-44):

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-  
Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Asn-Arg-Gln-  
Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Lys-Val-Arg-Leu

Porcine (1-44):

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-  
Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-  
Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Arg-Val-Arg-Leu

Human (1-44):

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-  
Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-  
Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu



moment. It has been reported that human GRH does not show species specificity in evoking responses among mammals, eg. human GRH is effective in inducing electrophysiological responses in rat pituitary cells (Chen, Barretto, Convey & Smith, 1989). The present results show that GRH has a species specificity between the frog and mammals (cow, pig and humans). It is possible that evolutionary changes occurred in the structures of GRHs and perhaps their receptors of these animals and that the frog is phylogenetically closer to the rat than the cow, pig or human.

The response profile of the GRH response, a slow outward current which decays, resembles that of other endogenous receptors in the *Xenopus* oocyte follicular layer, which suggests that it acts via a similar mechanism, namely via an increase in adenylate cyclase activity to produce a rise in cAMP and hence activation of  $K^+$  channels. That the GRH response is not due to the activation of endogenous receptors other than a specific GRH receptor is shown in by the action of known endogenous receptor antagonists. The latency of the response to GRH in *Xenopus* oocytes, at around 30 seconds to  $0.1\mu M$ , is not completely dissimilar to the GRH response induced in rat pituitary cells where the delay before the induction of the response was 1-5 minutes (Nussinovitch, 1988). The presence of such a long delay may be an indication that a second messenger is involved in the response to GRH in both the anterior pituitary cell and the *Xenopus* oocyte.

The results section shows that occasionally the slow outward current is preceded by a fast transient inward current. Such a fast transient current has also been reported for the adenosine response (Lotan Dascal, Cohen & Lass, 1982; Dascal, Landau & Lass, 1984). However, in a subsequent study by Stinnakre & Van Renterghem (1984) no such fast component was seen in the response to adenosine and it was suggested that this response may be due to a fast perfusion rate. Such a  $Cl^-$  current is generally induced by the release of calcium due to GRH induced activation of the PI pathway causing the formation of IP3 and DAG. If indeed a fast transient  $Cl^-$  component to the GRH response is real, it may be involved in the release of GH. It has been shown in cultured rat pituitary cells that phorbol esters, which mimic the action of DAG to activate PKC can stimulate GH release (Ohmura, Tsushima, Murakami *et al.*, 1984).

The reversal potential of the response, as with all the other slow outward currents induced by the activation of endogenous receptors, was around -100mV. This agrees with the expected reversal potential calculated for the

movement of  $K^+$  ions by Kusano, Miledi & Stinnakre (1982). The shift in the reversal potential seen in this study of 40mV is in agreement both with that expected from the Nernst equation and also that measured experimentally for the endogenous response to epinephrin (Kusano, Miledi & Stinnakre, 1982). Further evidence that the response to GRH is dependent on  $K^+$  came from the finding that the response could be inhibited by  $TEA^+$ . TEA has also been shown to abolish the current elicited by adenosine (Lotan *et al.*, 1982) and gonadotrophins (Woodward & Miledi, 1987 a).

A  $K^+$  current response may be a general property of the activation of the GRH receptor. In the whole cell-clamped rat anterior pituitary cell the application of GRH induced rhythmic outward currents, associated with an increase conductance to  $K^+$  when the cells were clamped at -40 mV (Nussinovitch, 1988).

The results have established that the response to GRH occurs in a dose-dependent manner (1nM- 1 $\mu$ M). Cultured ovine and bovine pituitary cells have also been shown to be responsive to GRH in a dose-dependent manner ( $10^{-12}$  -  $10^{-7}$ ) to produce a rise in GH release and also in the intracellular level of cAMP (Law, Ray & Wallis, 1984; Hart, Ray & Wallis, 1988). A role for cAMP is supported by the evidence showing that phosphodiesterase inhibitors, cyclic AMP derivatives and activators of adenylate cyclase can cause stimulation of growth hormone release (Schofield, 1967; Brazeau, Ling, Esch *et al.*, 1982). More direct evidence has come from observations that GRH can stimulate cAMP formation in cultured anterior pituitary cells of the rat (Bilezikjian & Vale, 1983) and of the sheep (Law, Ray, & Wallis, 1984) and can directly activate adenylate cyclase in pituitary membrane preparations (Harwood, Grewe & Aguilera, 1984). The slow inactivating response to GRH would also seem to be consistent with the pattern of cAMP increase in the ovine pituitary (Ray & Wallis, 1988), where there was a rapid 8- to 9- fold increase in the rate of GH release over 10-15min after which (15-30 min) the secretion rate declined slowly.

It is also known that cAMP (Brazeau, Ling, Esch *et al.*, 1982; Bilezikjian & Vale, 1983) and the adenylate cyclase system (Labrie, Gagne & Lefevre, 1983; Schettinni, Cronin, Hewlett, Thorner & Macleod, 1984) are involved in the secretory process of GH in response to GRH, and it would seem that the response to GRH in the *Xenopus* oocyte is a cAMP dependent process as the response to GRH resembles the response seen upon the injection of cAMP (Miledi & Woodward, 1989) and all the other endogenous responses which

give rise to an outward current response have been shown to be due to cAMP. The involvement of cAMP has also been shown by the facilitation of the response to GRH in the presence of forskolin, a similar facilitation in the endogenous  $\beta$ -adrenergic response was reported in *Xenopus* oocytes (Van Renterghem, Penit-Soria & Stinnakre, 1985)

From the results section it is apparent that the chelation of intracellular  $\text{Ca}^{2+}$  does not inhibit the response to GRH and therefore the response is not mediated by a release of intracellular calcium. In this respect the results are similar to those found for the response to adenosine. The injection of calcium has been shown to have no effect on the response to adenosine (Dascal, Lotan, Gillo *et al.*, 1985) and the injection of EGTA did not decrease the response, but facilitated it (Stinnakre & Van Renterghem, 1986). A similar facilitation of an endogenous receptor response by the injection of EGTA has also been observed for the response to gonadotrophins (Woodward & Miledi, 1987a)

#### **Functional significance of GRH receptors in follicle cells**

For some time now there has been some debate over the location of the endogenous receptors of the *Xenopus* oocyte. It has been established that defolliculation abolishes the response to GRH and to other endogenous responses. A logical conclusion to this may be that the receptor lies in the follicular layer and that defolliculation removes it. However, the possibility remains that the receptor is on the oocyte membrane but damaged by defolliculation. The present study does not solve this problem, but the effect of octanol (the gap-junction blocker) shows that the response is dependent on intact gap junctions between the oocyte and the follicular layer. It is reasonable to conclude that GRH receptors are found in follicle cells, which are coupled to oocytes via these gap junctions. If the receptors are indeed in the follicular cells, this might suggest that follicle cells play a role in controlling the development of oocytes by secreting growth hormone to oocytes. In mammalian oocytes, there is some evidence that an insulin-like growth factor (IGF-1) is involved in the growth and differentiation of granulosa cells, the granulosa cells having receptors for this growth factor (see Adashi, Resnick, D'Ercole *et al.*, 1985 for review). In addition it has been demonstrated that FSH is capable of upregulating IGF-1 binding sites in granulosa cells (Adashi, Resnick, Svoboda & Van Wyk, 1988) and that cAMP may play a role in this regard (Adashi, Resnick, Svoboda & Van Wyk, 1986). Thus a possible role for the GRH receptor on the follicular cell layer of *Xenopus* oocytes may have a similar role to IGF-1 receptors in mammalian granulosa cells.

## SECTION 9

### General Discussion

In this study, the expression of rat anterior pituitary gland receptors has been demonstrated in *Xenopus laevis* oocytes using intracellular recording and two-electrode voltage clamp to monitor the change in the membrane conductance activated by receptor-ligand binding. Using such electrophysiological techniques has allowed a study of the events which occur between the binding of the receptor to its ligand and the activation of ion channels. GnRH and the TRH receptors once expressed in the *Xenopus* oocyte cause the activation of a G-protein and the release of intracellular calcium from stores, which in turn causes the activation of a calcium-dependent Cl<sup>-</sup> current. The calcium ionophore, ionomycin, has proved to be a useful tool in the study of the intracellular calcium stores which are responsible for the membrane responses seen after receptor-ligand binding. The relevance of these findings to receptors in the pituitary gland and to the fertilization events in the *Xenopus* egg are discussed.

**The expression of exogenous GnRH and TRH receptors in *Xenopus laevis* oocytes; the relation to endogenous GnRH and TRH receptors in the anterior pituitary gland.**

The question which arises in a study where a receptor is being studied in a foreign environment is whether the response seen upon the activation of the receptor bears any resemblance or relevance to the receptor in its native cell.

There is evidence obtained from many systems to indicate that hormone and neurotransmitter receptors that mediate their effects through inositol phospholipid breakdown may be functionally coupled to phospholipase C by regulatory G-proteins. The modulation of binding of an agonist by guanyl nucleotides is characteristic of receptors that are coupled by GTP-binding proteins. The binding of a GnRH agonist in bovine anterior pituitary membranes is inhibited by guanyl nucleotides (Perrin, Haas, Porter et al., 1989). Further evidence for the involvement of G-proteins in the release of LH by GnRH comes from the finding that both GTP and the stable GTP analogue GTP $\gamma$ S provoke a dose-dependent release of LH and phosphoinositide turnover in permeabilised pituitary cells (Andrews, Staley, Huckle & Conn, 1986).

Pertussis toxin has been used to inhibit G-protein stimulated phosphoinositol turnover in some systems (Molski, Naccache, Marsh et al., 1984; Okajima & Ui, 1984). In the *Xenopus* oocyte, pertussis toxin inhibited the response to GnRH and TRH. However, pertussis toxin does not affect GnRH or guanine nucleotide-stimulated LH release (Conn, Morrelly, Dufau & Catt, 1979). Similarly, pertussis toxin is not effective on the action of TRH in GH3 cells (Martin, Lucus, Bajjalieh & Koalchyk, 1986). This does not mean that GnRH does not use a G-protein, as some G-proteins are unaffected by pertussis toxin, for example in hepatocytes (Lynch, Perpic, Blackmore & Exton, 1986).

Using gonadotrope enriched cultures, it has been shown that GnRH promotes a rapid (less than 1min) specific incorporation of  $^{32}\text{P}$  into the second messenger inositol trisphosphate (IP3) (Andrews & Conn, 1986). Also, GnRH was found to increase the rate of IP3 production for 60-90 min with a dose-dependency similar to that for LH release (Huckle & Conn, 1987). This finding is consistent with GnRH-stimulated hydrolysis by a phospholipase C-type reaction, which produces IP3 and diacylglycerol (DAG). It has been proposed that IP3 and DAG play a role in the gonadotrope. DAG acts as an activator of protein kinase C (Nishizuka, 1984) and IP3 provokes the release of  $\text{Ca}^{2+}$  from the intracellular  $\text{Ca}^{2+}$  store (Streb, Irvine & Berridge, 1983). Indeed, DAG production has been shown to rapidly rise on GnRH application

It has been shown (using the  $\text{Ca}^{2+}$ -sensitive dye quin-2) that there is a rise in intracellular  $\text{Ca}^{2+}$  in response to GnRH in gonadotropes (Clapper & Conn, 1985). Calcium has been shown to behave like a second messenger in pituitary gonadotropes (Marian & Conn, 1979). The use of the calcium ionophore ionomycin allowed the establishment of a clear relationship between the elevation of intracellular  $\text{Ca}^{2+}$  and the stimulation of gonadotrophin release (Conn, Kilpatrick & Kirsher, 1980). In the *Xenopus* oocyte the ability of  $\text{Ca}^{2+}$  stores to release  $\text{Ca}^{2+}$  was shown to be essential for the response to GnRH and TRH to continue, as a blocker of intracellular  $\text{Ca}^{2+}$  release TMB-8 abolished the response. However in the gonadotrope by contrast, stabilization of the intracellular store does not interfere with the ongoing stimulation of release of LH by GnRH (Bates & Conn, 1984). Although, more recently a study by Hansen, McArdle & Conn (1987) has suggested a potential role for intracellular  $\text{Ca}^{2+}$  in very early LH release, a component of which may be mediated by intracellular  $\text{Ca}^{2+}$  stores.



An apparent difference between the response to GnRH in the pituitary and that in the *Xenopus* oocyte expressing GnRH receptors is involvement of extracellular  $\text{Ca}^{2+}$  in the response. It has been shown that GnRH in the pituitary binds to the receptor and induces an increase in the intracellular  $\text{Ca}^{2+}$  concentration via the release of  $\text{Ca}^{2+}$  from internal stores and via influx of  $\text{Ca}^{2+}$  from the extracellular solution (Limor et al., 1987). Unlike the *Xenopus* oocyte, where removal of extracellular  $\text{Ca}^{2+}$  does not appear to inhibit the response to GnRH, when  $\text{Ca}^{2+}$  is omitted from the bathing medium of cultured gonadotropes the cells become refractory to GnRH (Marian & Conn, 1979). Similarly, in gonadotropes removal of  $\text{Ca}^{2+}$  results in prompt extinction of the response (Bates & Conn, 1984).

A further difference in the response to GnRH in the pituitary and the *Xenopus* oocyte is the ion channel which is activated by an increase in the intracellular concentration of  $\text{Ca}^{2+}$ . In the *Xenopus* oocyte a rise in intracellular  $\text{Ca}^{2+}$  causes the opening of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. The mechanism of the GnRH response in ovine (Mason & Waring, 1986) and rat (Croxtan et al., 1988) pituitary cells has also been studied. GnRH receptors in the gonadotropes of these species operate via the activation of a  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel. However, it is interesting to note that the dose-dependency of the response in the present study (1nM-1 $\mu$ M) is similar to that seen in the pituitary for the release of LH (Liu & Jackson, 1979), suggesting that expressed GnRH receptors retain their original binding affinity for GnRH.

Thus it is apparent that the expressed GnRH receptor in the *Xenopus* oocyte causes the activation of a different G-protein linked pathway from that in the pituitary. It would appear that the pathway activated is endogenous to the oocyte and the receptor is able to activate the G-protein which is already in the membrane which cause the activation of the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channel. The next question which arises is why the oocyte has an endogenous G-protein and second messenger system which causes a rise in intracellular  $\text{Ca}^{2+}$  and opens  $\text{Cl}^-$  channels.

**The  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current activated by exogenous receptors; the relation to fertilization events in the *Xenopus* egg and the intracellular calcium stores.**

The present study has shown that the activation of exogenous GnRH and TRH receptors in the *Xenopus laevis* oocyte membrane causes the opening of calcium activated chloride channels. Similar results have been found for the expression of serotonin, substance K, acetylcholine, glutamate and many other neurotransmitter and hormone receptors (see Dascal 1987 and Snutch 1988 for review). The currents elicited by these agonists all show appreciable latencies. The similarities in the response latencies probably arise because the different exogenous and endogenous receptors use a common endogenous signalling pathway in the oocyte (Parker, Sumikawa & Miledi, 1987; Takahashi, Neher & Sakmann, 1987). Further experiments showed that this signalling pathway operates through phosphoinositide metabolism and calcium release in a similar manner to that described in other cell types (Berridge & Irvine 1984; Hokin 1985; Berridge, 1986, 1987).

The activation of the egg by sperm is similar to interactions of neurotransmitters and hormones with membrane receptors; in particular, fertilization appears to activate a G-protein leading to  $\text{IP}_3$  production and  $\text{Ca}^{2+}$  release (Kline & Jaffe, 1979). Indeed Kline, Simoncini, Mandel et al., (1988) suggest that there is a sperm protein receptor which causes the activation of a G-protein. The release of  $\text{Ca}^{2+}$  causes diverse responses in the egg, including ion channel opening and cortical granule exocytosis and endocytosis (Kline, 1988). The main effect of the binding of sperm to the egg is to cause a fast depolarization of the membrane potential which is thought to prevent polyspermy (Cross & Ellison, 1980; Jaffe, Cross & Picherel, 1983). Such a depolarisation is common in amphibians and it has been known for some years now that the current underlying this fertilization potential is mostly carried by an efflux of  $\text{Cl}^-$  (Ito, 1972)

It has also been shown that a wave of  $\text{Ca}^{2+}$  occurs at fertilization. The  $[\text{Ca}^{2+}]_i$  first increases at the point of sperm entry and spreads throughout the cytoplasm in the sea urchin, *Xenopus* and hamster egg (Jaffe, 1983; Kubato et al., 1987; Miyazaki et al., 1986). This wave of Ca can also be induced by localized injection of  $\text{IP}_3$  into the sea urchin and *Xenopus* oocyte (Swann & Whittaker, 1986; Busa et al., 1985). Very recently a similar finding has been reported upon

the binding of substance K to its receptor once expressed in the *Xenopus* oocyte (Brooker, Seki, Croll & Wahlestedt, 1990). When oocytes were incubated with the blocker of intracellular  $\text{Ca}^{2+}$ -release from stores TMB-8, which was found to be effective in blocking the response to GnRH and TRH in the present study, it was found that the activation potential was also blocked (Kline & Nuccitelli, 1985). This finding suggests that the mobilisation of the same intracellular stores are responsible for the response to both expressed receptor activation and fertilization. Thus the fertilization potential would appear to be caused by the opening of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels.

There are several pieces of evidence to suggest that the  $\text{Cl}^-$  channels activated by fertilization in the egg are similar to those activated by a rise in intracellular  $\text{Ca}^{2+}$  in the oocyte, in particular, the single channel conductance is similar (Jaffe, Kado & Muncy, 1985; Dascal, 1987) and there is smaller current elicited in the vegetal hemisphere than the animal, as measured by extracellular current recording (Jaffe, Kado & Muncy, 1985; Kline & Nuccitelli, 1985). Further evidence that the activation of expressed receptors causes the same event which occur during fertilization come from a study by Kline, Simoncini, Mandel et al., (1988). They showed that the activation of serotonin or acetylcholine receptors in *Xenopus* eggs caused a similar membrane depolarisation and exocytosis to that induced by sperm.

Therefore the functional expression of the GnRH and TRH receptors leads to the activation of a native second messenger system which is essential to the egg for fertilization and to prevent polyspermy.

### **The uses of *Xenopus* oocytes**

This study has shown the versatility of the *Xenopus* oocyte, as various types of study can be carried out on these cells. These oocytes are useful for the study of endogenous receptors, calcium stores and the expression of exogenous receptors by the injection of foreign mRNA.

The present study has shown that *Xenopus* oocytes are capable of expressing a variety of anterior pituitary receptors and that the measurement of the resultant  $\text{Cl}^-$  currents which are evoked by the binding of an exogenous receptor with its ligand are a sensitive means of testing for the presence of receptors in the oocyte membrane. The success rate for the expression of GnRH and TRH receptors from rat pituitary mRNA was quite high (64% for GnRH and 84% for TRH). As mentioned previously one of the eventual aims of this study is to use the



*Xenopus* oocyte system to clone the GnRH and TRH receptors. Such an approach has proved successful for the cloning of the serotonin (5-HT<sub>1c</sub>) and substance K receptors (Julius, MacDermott, Axel et al., 1988; Masu, Nakayama, Tamaki et al., 1987). The initial attempts to clone the GnRH receptor were unsuccessful and if one considers the 5-HT<sub>1c</sub> receptor cloning a few possible reasons for our difficulty become apparent. 5-HT mRNA was extracted from a tissue (choroid plexus) where the expression of such receptors is high. The response to 5-HT after the injection of mRNA was large, in the  $\mu$ A order (Julius, MacDermott, Axel et al., 1988). In the case of GnRH the source tissue has a relatively low density of GnRH receptors and therefore the responses to GnRH were in the nA order when expressed in *Xenopus* oocytes. Therefore when a complementary DNA library for the serotonin receptor was made a high number of clones for this receptor were formed. However, recently a cell line which expresses a GnRH receptors with greater abundance than in the pituitary has been developed (Sealfon et al., 1990). The use of such a cell line to construct a complementary DNA library may increase the abundance of GnRH clones and hence aid the search for the clone for the GnRH receptor.

During the course of these studies it has become apparent that whilst electrophysiology forms a very sensitive means of testing for the presence of an expressed receptor there are certain drawbacks. Firstly, with such an invasive technique, it takes some time for each oocyte to seal before study and it is only practical to study one, or at most two oocytes at once. Hence the screening of a cDNA library is very time-consuming. More recently it has become apparent that measurement of  $[Ca^{2+}]_i$  using the fluorescent indicators fura-2 or fluo-3 may be a better means of screening a large number of oocytes, as more than one oocyte can be tested at once (Brooker, Seki, Croll & Wahlestedt, 1990).

Another consideration which must be taken into account is that not all receptors activate a G-protein which causes a rise in intracellular  $Ca^{2+}$  and hence open a Cl-channel in *Xenopus* oocytes. Some receptors are linked to G-proteins which stimulate adenylate cyclase to cause a rise in the cAMP level, for example the  $\beta$ 2- adrenergic receptor (Kolbilka, MacGregor, Daniel et al., 1987). Other receptors are linked to a G-protein which inhibits adenylate cyclase and therefore causes a decrease in intracellular cAMP (White & Reisine, 1990). In a defolliculated oocyte, a change in the intracellular concentration of cAMP does not cause any change in the conductance of the oocyte membrane and hence electrophysiology cannot be used to study the expression of such receptors.

However, if radioimmunoassay of cAMP is performed, it is possible to study expression of such receptors in *Xenopus* oocytes (White & Reisine, 1990).

### **Maturation of *Xenopus* oocytes and the involvement of the follicular layer.**

The present study has revealed the presence of endogenous receptors for growth-hormone releasing hormone (GRH). Such a finding prompts the question of their function.

It would appear from several studies that follicular cells are important for oocyte maturation. When follicular oocytes of *Rana pipiens* were incubated with gonadotrophins, the oocytes matured (Heilbrunn, Daughert & Wilbur, 1939). However, when oocytes were defolliculated, this maturation in the presence of gonadotrophins was prevented, but defolliculated oocytes matured in response to progesterone (Masui, 1967). This discovery led to the hypothesis that gonadotrophins induce follicular cells to secrete a progesterone-like hormone which induces maturation, this was later shown to be the case by Fortune, Concannon & Hansel (1976). Thus it was established that follicle cells play some role in the maturation of oocytes.

It is interesting to note that <sup>oocytes</sup> which are growing rarely respond to maturation signals from outside. Indeed, Reynnhaut, Taddai, Smith & LaMarca (1975) reported that follicle enclosed oocytes of *Xenopus* must be at least 1.2mm in diameter to be responsive to gonadotrophin-stimulation. However, if smaller oocytes (less than 0.9mm) have their follicular layers removed, they can be induced to mature by progesterone. Hence, for smaller oocytes, there appears to be some inhibiting factor which prevents oocyte maturation, whilst the oocyte is still young. It is known that progesterone <sup>a</sup> rises intracellular  $Ca^{2+}$  concentration and that this rise in  $Ca^{2+}_i$  is important in maturation, since injection of the  $Ca^{2+}$  chelator EGTA, prevents progesterone induced maturation (Masui et al., 1977). Thus in smaller oocytes, the possibility arises that the follicular layer may inhibit the action of progesterone by reducing the rise in intracellular  $Ca^{2+}$ . Such an inhibition of a rise in intracellular in *Xenopus*, is caused by a rise in cAMP, as this inhibits the maturation by progesterone (O'Connor & Smith, 1976; Godeau et al., 1977). Thus, the finding, in the present study that GRH receptor activation in the follicular layer causes a rise in cAMP in the oocyte, by passing through gap junctions, may be one of the

controlling factors in the maturation of oocytes and such a topic would benefit from further study.

"In research, the horizon recedes as we advance.....  
And research is always incomplete."

Mark Patterson

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## APPENDIX

## STATISTICAL TESTS

In the present study where the number of observations was smaller than 30, the  $t$  test, one of parametric tests, was not suitable for the statistical test because it required normal distributions and homogeneity of variations which could not be easily checked. The Mann-Whitney U test, one of nonparametric tests (or distribution-free statistics), for the statistical test for two independent groups was used. This test needed only nominal or ordinal scale data and not normal distribution or homogeneity of variations (Phillips, 1978). Although a one-tailed test could be applied to some cases where we could predict that the difference would be in a specific direction, two-tailed tests were generally used in the present study

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# Functional expression of rat pituitary gonadotrophin-releasing hormone receptors in *Xenopus* oocytes

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## ABSTRACT

Expression of receptors for the hypothalamic regulatory peptide, gonadotrophin-releasing hormone (GnRH), was investigated by intracellular recording from *Xenopus* oocytes injected with poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands. Membrane depolarizations were induced in oocytes in a dose-dependent fashion following the application of GnRH (10nM - 1μM) or a GnRH superactive agonist, buserelin (1nM - 1μM). The response was reversibly blocked by the addition of a GnRH antagonist (1μM). TRH (10nM - 1μM) had no effect on most of these oocytes. In

contrast, some other oocytes which showed no responses to GnRH or to the GnRH agonist, displayed depolarizing responses to TRH (10nM - 1μM). A relatively small number of oocytes responded to both ligands. Control oocytes did not respond to the GnRH analogues or to TRH. This successful expression of the GnRH receptor could provide a new approach to the study of the receptor, and serve as a means for the isolation and cloning of the encoding genes.

## INTRODUCTION

The pituitary receptor for GnRH has been the focus of considerable attention due to the importance of GnRH analogues in the management of a variety of reproductive disorders, including cancer of the breast and prostate (Nicholson, Walker, Turkes *et al.* 1984; Parmar, Phillips, Lightman, *et al.* 1985; Schally & Comaru-Schally, 1987). Under physiological conditions, GnRH activates membrane-bound receptors on pituitary gonadotrophs, and sets in motion a sequence of intracellular events that culminate in the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH). These GnRH receptors display both up- and down-regulation and change dynamically in number in response to changing patterns of exposure to GnRH (Naik, Saade, Datta & Clayton, 1985). Intermittent application of GnRH or GnRH superagonists is now routinely used to stimulate gonadotrophin secretion in the treatment of infertility. Chronic exposure to pharmacological doses of these compounds, on the other hand, leads to decreased gonadotrophin secretion, and that effectively eliminates the gonadal secretion of steroid hormones. Such reversible, chemical castration has considerable value in the therapeutic management of sex-steroid-dependent tumours. Further progress in understanding these receptor mechanisms is limited by lack of structural information, the lability of the receptor in solution and the low level of expression in pituitary tissue.

The thyrotrophin-releasing hormone (TRH) receptor has recently been expressed in the *Xenopus* oocyte following the injection of mRNA from the GH3 cell line (Oron, Gillo, Straub & Gershengorn, 1987; Meyerhof, Morley, Schwarz & Richter, 1988) and the mechanisms involved in down-regulation evaluated electrophysiologically (Oron, Straub, Traktman & Gershengorn, 1987). The GH3 rat pituitary tumour cell line, which expresses the TRH receptor, has provided a convenient source of material for the study of TRH receptor regulation. Unfortunately, there are no established cell lines that express specific high-affinity receptors for GnRH. The current study demonstrates the expression of functional GnRH receptors following the injection of rat pituitary mRNA into *Xenopus* oocytes. TRH receptor expression was also observed in some oocytes.

## MATERIALS AND METHODS

### Peptides

The following peptides were used as receptor ligands: GnRH and the GnRH agonist, buserelin ([D-Ser(But)<sup>6</sup>, Pro<sup>9</sup>-N-ethylamide] GnRH) from Hoechst AG, Frankfurt, F.R.G.; GnRH antagonist [N-Ac-D-2-naphthylalanine<sup>1</sup>, D-pCl-Phe<sup>2</sup>, D-Trp<sup>3</sup>, D-diethyl hArg<sup>6</sup>, D-Ala<sup>10</sup>] GnRH, was a gift from Syntex Research, Palo Alto, CA, USA. TRH was supplied by Cambridge Research Biochemicals, Cambridge, U.K.

### Preparation of poly(A)<sup>+</sup> mRNA from rat pituitaries

Anterior rat pituitary glands were removed from adult female rats of the Sprague-Dawley strain, and a preparation of total RNA extracted following the method of Chomczynski & Sacchi (1987). A poly(A)<sup>+</sup>-enriched mRNA fraction was obtained by oligo(dT) affinity chromatography (Maniatis, Fritsch & Sambrook, 1985) and dissolved in sterile deionised water to a concentration of approximately 1mg/ml. Several batches of poly(A)<sup>+</sup> mRNA preparations were used for experiments.

### Microinjection of mRNA into *Xenopus* oocytes

Oocytes were removed from adult *Xenopus laevis*, dissected from their surrounding membranes, and maintained overnight at 19°C in modified Barth's medium (Aoshima, Iio, Anan *et al.* 1987) containing penicillin (100 u/ml), streptomycin (100 μg/ml) and neomycin (100 μg/ml). 50 ng of the poly(A)<sup>+</sup> mRNA preparation dissolved in 50nl of water was injected into each oocyte. The oocytes were incubated in modified Barth's medium for 48h at 19°C before evaluation. Control oocytes were either untreated or injected with 50nl of water.

### Electrophysiological recordings

Electrophysiological measurements were performed using

conventional intracellular recording techniques (Georgiou, House, McNiven & Yoshida, 1988). A fine, inert nylon mesh was used to secure the oocyte during impalement, and during exchange of media. A glass microelectrode, filled with 3M KCl (resistance 20-50 MΩ), was inserted into the oocyte for the measurement of the membrane potential. A second intracellular microelectrode (20-40 MΩ) was inserted to administer current for the measurement of the membrane resistance. Only oocytes (either control or mRNA-injected) showing resting membrane potentials more negative than -50 mV were used. The average resting potential and the input resistance values were -63 mV and 1.4 MΩ respectively. All experiments were performed at room temperature (20-23°C).

RESULTS

GnRH and GnRH agonist caused marked membrane electrical responses in some oocytes injected with poly(A)<sup>+</sup> mRNA prepared from rat anterior pituitary tissue. An example is shown in Fig. 1, and involves the application of GnRH, the GnRH agonist and TRH.

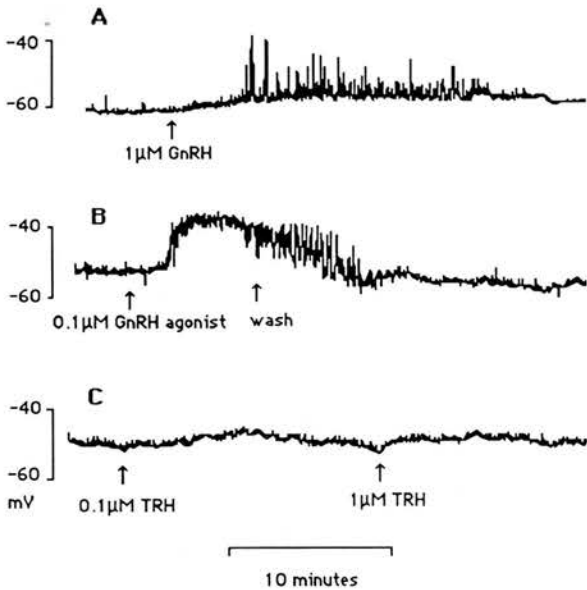


Fig.1. Typical membrane voltage responses of *Xenopus* oocytes injected with 50ng of poly(A)<sup>+</sup> RNA prepared from rat pituitary gland. (A) 1μM GnRH, (B) 0.1μM GnRH agonist (buserelin) and (C), 0.1μM and 1μM TRH were added to the medium (115mM NaCl, 1mM KCl, 1.8mM CaCl<sub>2</sub> and 10mM HEPES, at pH 7.4) bathing the oocyte. The arrows indicate the onset of ligand administration to the chamber, or the commencement of its removal.

Numerous episodes of depolarization (5-15 mV), were observed to commence 1-5 min after the continuous application of GnRH (1 μM) to the bathing medium. These depolarizations slowly declined in amplitude and incidence over the following 10-15 min. Fig. 1B shows that the subsequent application of the GnRH agonist to this oocyte, at the lower concentration of 0.1 μM, caused a dramatically enhanced response and resulted in a sustained depolarization of around -30 mV. The latency of the response was shortened and the size and frequency of depolarizations became larger when the agonist was applied to the same oocyte. This response was progressively reduced by washing out the agonist, or by desensitization over a period of some minutes. During this recovery phase, the oocyte showed individual depolarizations as was

observed in the case of GnRH application. In contrast, no response was observed from this oocyte when exposed to 0.1 μM or 1.0 μM TRH (Fig. 1C). However, further depolarizations of up to 15 mins in duration were observed when either GnRH or its agonist was repeatedly added to and removed from the bathing medium. The membrane depolarizations induced by GnRH and the GnRH agonist were eliminated or reversed by the application of the GnRH antagonist, according to the time of administration. An example is

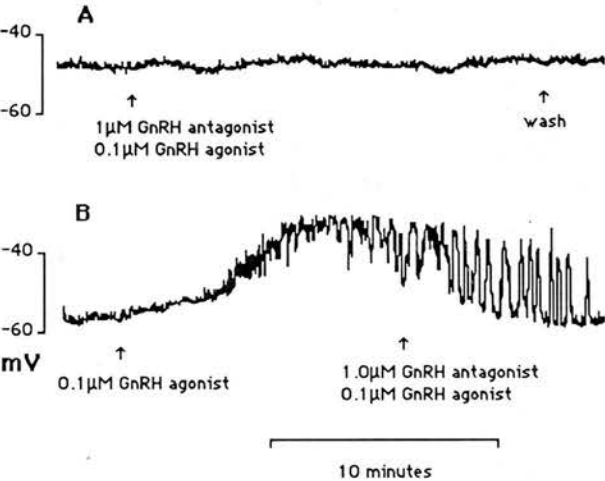


Fig. 2. Inhibition by a GnRH antagonist of GnRH agonist-induced responses of *Xenopus* oocytes injected with 50ng of poly(A)<sup>+</sup> RNA prepared from rat pituitary gland. (A), the arrow indicates the application of GnRH agonist (0.1μM) to the mRNA-injected oocyte in the presence of the GnRH antagonist (1.0 μM), and (B) shows the application of GnRH antagonist during GnRH agonist-induced responses. The peptides were added to the medium (115mM NaCl, 1mM KCl, 1.8mM CaCl<sub>2</sub> and 10mM HEPES, at pH 7.4) bathing the oocyte.

illustrated in Fig. 2. In this recording of a GnRH responsive oocyte, the GnRH antagonist (1.0 μM) produced a complete inhibition of the effect produced by the GnRH agonist (0.1 μM) when administered simultaneously (Fig. 2A). No effect was observed when antagonist alone was applied to oocytes (not shown). The antagonist abolished the response when administered during a period of agonist-induced depolarizations. Clearly defined waves of depolarization were revealed as the antagonist started to compete with the agonist-induced depolarization plateau (Fig. 2B). The GnRH or GnRH agonist showed a dose-dependent response, as the depolarizing membrane fluctuations became larger in size and appeared more frequently with increasing ligand concentration. It is interesting that some mRNA-injected oocytes, which did not respond to GnRH or its agonist, responded to TRH, regardless of the order in which GnRH or TRH were added to the bathing medium. In these oocytes, TRH induced significant depolarizing responses in a dose-dependent manner and Fig. 3 illustrates a typical example of such an oocyte. Initial application of TRH (0.1 μM) induced a continuous membrane depolarization which appeared very rapidly (within 10 s), the effect decaying with time (Fig. 3A). Subsequent application of GnRH (1μM) and GnRH agonist (0.1 and 1μM) produced no effect (Fig. 3B), but the oocyte remained responsive to TRH (0.1μM) albeit with a longer latency, and with

smaller and less frequent depolarizations (Fig. 3C). A small number of oocytes showed responses to both ligands.

Sixty-five percent (15/23) of mRNA-injected oocytes responded to the peptide ligand. The membrane activity of control oocytes, either untreated or those injected with water, was unaffected by the application of 0.1  $\mu$ M to 1.0  $\mu$ M concentrations of GnRH, GnRH agonist, GnRH antagonist or TRH (results not shown).

## DISCUSSION

The present data show that poly(A)<sup>+</sup> mRNA obtained from the rat pituitary gland and encoding for hypothalamic peptide receptors, including the receptor for GnRH, can be translated in *Xenopus* oocytes. Furthermore, the GnRH receptor is incorporated into the plasma membrane in such a way as to generate a measurable biological response. GnRH or the GnRH agonist induced membrane depolarizations in some oocytes in a dose-dependent fashion, and these responses were blocked by the application of a GnRH antagonist, providing evidence for the involvement of a specific receptor-binding site. The GnRH receptor translated in the oocyte appeared to have some pharmacological kinetics that resemble those of the native GnRH receptor in the rat pituitary gland. The agonist used in these studies is at least 100-fold more potent than GnRH in activating the GnRH receptor of the pituitary gland (Friedrich, Etzrodt, Becker *et al.* 1978). A not dissimilar difference

TRH responsiveness, presumably due to receptor occupancy. Most oocytes failed to respond to GnRH following exposure to the antagonist, despite extensive washing, presumably due to the high affinity of the antagonist for the receptor. Responses to GnRH were observed to continue in some instances for periods in excess of 15 minutes, though the absolute duration of the response was not explored. Considerable variation between oocytes was apparent in the duration and latency of the response within this recording period. The latency varied with the concentration of the applied ligand (higher concentrations producing shorter latencies) and with desensitisation of the oocyte. Latency lengthened with repeated applications. Some oocytes responded to the repeated addition of ligand; the ligand solution having been removed and replaced after an interval of approximately 15 minutes. However, other oocytes responded once only, or became progressively more refractory with repeated administration of the peptides. GnRH and TRH were applied to the same oocyte and it was found that most of the mRNA-injected *Xenopus* oocytes seemed to respond to either GnRH or TRH, but not usually to both in the same oocyte. This was irrespective of the order in which the ligands were administered or the time of analysis relative to the injection of the message. Oocytes expressing TRH receptors were more frequently encountered than those expressing GnRH receptors. This might be because the TRH receptor mRNA shows greater stability in this system. Co-expression of GnRH and TRH receptors following mRNA injection was, however, observed in some oocytes.

The potential of the *Xenopus* oocyte to construct functional membrane-bound receptors has been established in the study of several neurotransmitters following the injection of total mRNAs extracted from different tissues (Aoshima *et al.* 1987; Verdoorn, Kleckner & Dingledine, 1987) and purified mRNA transcribed *in vitro* from cDNA constructs (Kubo, Fukuda, Mikami *et al.* 1986; Schofield, Darlison, Fujita *et al.* 1987; Masu, Nakayama, Tamaki *et al.* 1987). The responsiveness of the mRNA-injected oocyte may depend on the capability of the transcribed receptor to interact with an endogenous second messenger system. Both GnRH- and TRH-induced secretion of pituitary hormones probably involve such second messengers as 1,2-diacylglycerol, inositol 1,4,5-trisphosphate and calcium ions (Conn, 1986; Chang, McCoy, Graeter *et al.* 1986; Gershengorn, 1986), whilst a G-protein is thought to be functionally associated with GnRH receptor-mediated events (Andrews, Staley, Huckle & Conn, 1986). An important question now arises as to whether the ligand-induced phenomena reported here are coupled to a *Xenopus* oocyte second messenger system or an exogenous system introduced by the mRNA from the pituitary gland. The *Xenopus* oocyte has an active phosphoinositide-protein kinase C system, and numerous membrane bound ion channels (Oron, Dascal, Nadler & Lupu, 1985). GTP-binding regulatory proteins in *Xenopus* oocytes have been shown to act as intermediates in the membrane current responses to substances such as glutamate following injections of mRNA derived from mammalian brain (Sugiyama, Ito & Hirono, 1987). Thus it does appear that the responses observed could have been mediated via a *Xenopus* intracellular signalling mechanism. This is an important consideration if the system described here is to be used for screening mRNA derived from putative cDNA clones of specific receptors.

The mechanisms by which these second messenger events mediate the ionic changes that have been recorded in this study remain to be elucidated, but they presumably relate to the opening of a number of ion channels. The response mechanisms of GnRH and TRH receptors may differ in ionic dependency and studies are in progress to characterise these ionic events in terms of channel conductances.

In the light of these findings, it should now be possible to use the *Xenopus* oocyte as a model for studying the GnRH receptor, and other similar receptors, in terms of their ligand-induced activation and coupling to second messenger systems. The receptor expressed in this way is isolated from its normal milieu in the pituitary and is not subject to the influence of the endogenous environment. Use of this model should permit us to distinguish between the properties of the receptor itself and those which are due to pituitary-specific second messenger systems.

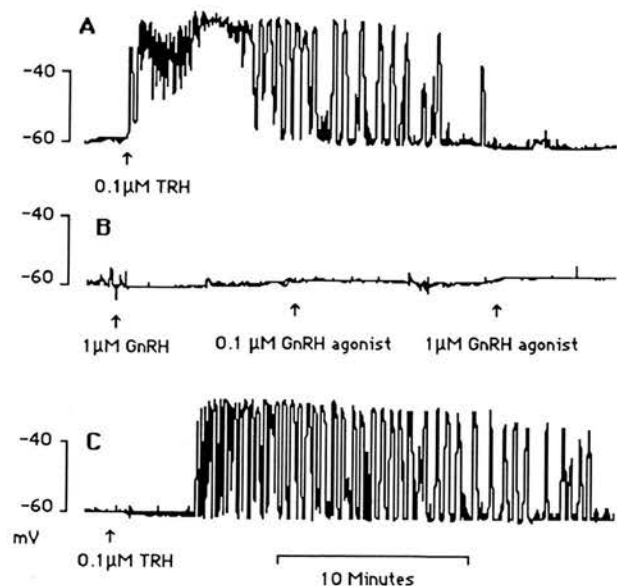


Fig. 3. Membrane voltage responses induced by TRH in an oocyte injected with 50 ng of rat pituitary poly(A)<sup>+</sup> RNA. The oocyte responded to 0.1  $\mu$ M TRH (A), but did not respond to 1.0  $\mu$ M GnRH or to 0.1 or 1.0  $\mu$ M GnRH agonist (B). The oocyte remained responsive to 0.1  $\mu$ M TRH (C). The peptides were added to the medium (115 mM NaCl, 1 mM KCl, 1.8 mM CaCl<sub>2</sub> and 10 mM HEPES, at pH 7.4) bathing the oocyte.

in potency was observed in the current studies. Likewise, the action of GnRH or its agonist was inhibited by the GnRH receptor antagonist. Some desensitization was observed in both GnRH and

This oocyte expression system might also be used as part of a strategy for the cloning of the receptor cDNA. It is difficult to isolate sufficient pure receptor protein to permit the partial sequence analysis necessary to construct a probe required for the conventional cloning approach. This sensitive electrophysiological assay in oocytes could be used to screen pituitary cDNA libraries to isolate a functional cDNA clone encoding the GnRH receptor.

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## Activation of gonadotrophin-releasing hormone receptors expressed in *Xenopus laevis* oocytes induces a chloride current

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In this study, messenger RNA from the rat pituitary gland, expressing gonadotrophin-releasing hormone (GnRH) receptors, was injected into *Xenopus* oocytes. With conventional intracellular recording, the response to GnRH or its agonist buserelin (1 nM–1  $\mu$ M) has been shown to consist of a sustained membrane depolarization with superimposed potential fluctuations (Eidne *et al.* 1988).

Whole-cell current was measured using two-electrode voltage-clamp techniques to clamp the membrane at the resting potential (–60 mV). The application of GnRH or buserelin resulted in a wave-like inward current (Fig. 1A), which was blocked by the GnRH antagonist [N-Ac-D-2-naphthylalanine<sup>1</sup>, D-pCl-Phe<sup>2</sup>, D-Trp<sup>3</sup>, D-diethyl-hArg<sup>6</sup>, D-Ala<sup>10</sup>] GnRH. This inward current reversed at around –20 mV (Fig. 1B), which is close to the chloride equilibrium potential measured in *Xenopus* oocytes (see Dascal, 1987). This reversal potential was shifted by changing the external Cl<sup>–</sup> concentration as predicted for Cl<sup>–</sup> electrode. The addition of Cl<sup>–</sup> channel blockers, 9-AC (9-anthracene carboxylic acid; 1–2 mM) and DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 0.1 mM) inhibited the GnRH response. Hence the GnRH response elicited from *Xenopus* oocytes after injection of rat pituitary mRNA is mediated by a chloride current.

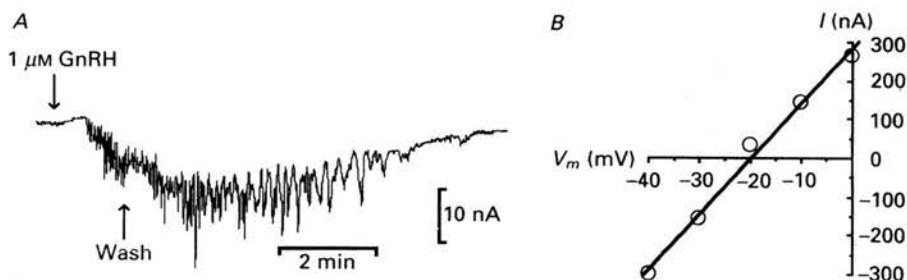


Fig. 1. A, whole-cell current response induced by GnRH (1  $\mu$ M) in a *Xenopus* oocyte expressing exogenous GnRH receptors, bathed in standard solution (115 mM-NaCl, 1 mM-KCl, 1.8 mM-CaCl<sub>2</sub>, 10 mM-HEPES-NaOH, pH 7.4). B, Current-voltage relationship of a GnRH response from an oocyte bathed in standard solution.

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# Chloride Channels Mediate the Response to Gonadotropin-Releasing Hormone (GnRH) in *Xenopus* Oocytes Injected with Rat Anterior Pituitary mRNA

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Functional expression of receptors for GnRH was studied using *Xenopus laevis* oocytes injected with poly(A)<sup>+</sup> mRNA extracted from rat anterior pituitary glands. Whole-cell currents were monitored using two-electrode voltage-clamp techniques. In oocytes which responded to both GnRH and TRH, the GnRH response showed a longer latency and time-to-peak than the TRH response. The response to GnRH or an agonist of GnRH receptors, buserelin (1 nM–1  $\mu$ M) consisted of current fluctuations and occurred in a dose-dependent manner. This GnRH response was blocked by the Cl<sup>-</sup> channel blockers 9-AC (9-anthracene carboxylic acid; 1 mM), 4,4'-diisothiocyanastilbene-2,2'-disulfonic acid (0.1 mM), and diphenylamine-2-carboxylic acid (0.1 mM). The reversal potential for the GnRH-induced current fluctuations was -25 mV, comparable with the reported Cl<sup>-</sup> equilibrium potential in *Xenopus* oocytes, and its shift, when the external concentration of Cl<sup>-</sup> was changed, was reasonably described by the Nernst equation. These results indicate that the GnRH-induced response was dependent on the activity of Cl<sup>-</sup> channels. Ca<sup>2+</sup> also plays a role, as the GnRH-induced response was reversibly suppressed by a calmodulin inhibitor, chlordiazepoxide (0.2  $\mu$ M), and by a blocker of intracellular Ca<sup>2+</sup> release, TMB-8 (8-(N,N-diethylamino) octyl-3,4,5-trimethoxybenzoate; 0.1–0.2 mM). It is concluded that GnRH (and TRH) receptors, expressed in *Xenopus* oocytes by injecting exogenous mRNA from rat anterior pituitary glands, operate via activation of Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels. (Molecular Endocrinology 3: 1953–1960, 1989)

## INTRODUCTION

GnRH is an important peptide known to stimulate the synthesis and release of LH and FSH in anterior pituitary

cells. GnRH agonists are widely used in clinical practice to treat hormone-dependent diseases such as breast cancer, endometriosis, prostatic cancer, etc. (1–3). Further progress in this area would be greatly assisted by a knowledge of the molecular structure and functional properties of the GnRH receptor. The direct study of the neuropeptide receptors is, however, difficult owing to their lability and low concentration in the lipid bilayer. The technique of expressing foreign mRNA in *Xenopus laevis* oocytes (4, 5) has made it possible to translate exogenous mRNA encoding receptors and ion channels. *Xenopus* oocytes are large in size and very efficient in synthesizing exogenous receptors (see Refs. 6–8 for review). Among others, the receptor for TRH has been studied by injecting mRNA derived from rat GH<sub>3</sub> pituitary tumor cells which are known to express TRH receptors (9–11). *Xenopus* oocytes have been successfully used as part of a cloning strategy to isolate several receptors (12, 13). We have previously demonstrated functional expression of GnRH receptors in *Xenopus* oocytes which were injected with poly(A)<sup>+</sup> mRNA isolated from rat pituitary glands using intracellular-recording techniques (14). In such oocytes, depolarizing potential fluctuations were induced in a dose-dependent manner in response to GnRH or an agonist of GnRH receptors, buserelin. The present study was carried out to characterize the ionic mechanisms of the GnRH response in *Xenopus* oocytes by measuring whole-cell currents using two-electrode voltage-clamp techniques.

## RESULTS

### Current Responses Induced by GnRH and by TRH

Control (uninjected or water-injected) *Xenopus* oocytes showed no changes in membrane current when exposed to GnRH, buserelin (a GnRH receptor agonist),

or TRH at concentrations ranging from 1 nM to 1  $\mu$ M (not illustrated). Responses were observed only in oocytes injected with poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands after the application of GnRH or TRH (Fig. 1). Perfusion of GnRH (1  $\mu$ M) into the external solution induced a slow, long-lasting inward current with superimposed fluctuations appearing with a latency of approximately 50 sec, reaching its peak about 260 sec after the application of peptide (Fig. 1A). A response to TRH was recorded from the same oocyte (Fig. 1B), which also consisted of a continuous inward current with superimposed current fluctuations. In both GnRH and TRH responses, current fluctuations became less frequent and smaller in size on washing out the peptide. As reported in the previous paper (14), some oocytes responded to both GnRH and TRH. In this study, the total number of successful recordings of oocytes which were tested both with GnRH and TRH was 25. Four oocytes (16%) responded to GnRH only, nine (36%) to TRH only, and 12 (48%) responded both to GnRH and TRH. There were characteristic differences between the GnRH and TRH responses obtained from the same oocytes. Both the latency and the time-to-peak of the GnRH response were always longer than those of the TRH response. The latency and the time-to-peak of the responses in the oocyte illustrated in Fig. 1 were 50 and 260 sec, respectively, for GnRH whereas the values were 15 and 95 sec for TRH. The mean values of the latency and the time-to-peak obtained from six mRNA-injected oocytes were  $56 \pm 32$  sec (mean  $\pm$  sd) and  $194 \pm 59$  sec, respectively, for the GnRH response and  $26 \pm 19$  sec and  $92 \pm 43$  sec for the TRH. GnRH responses exhibited significantly larger values for both latency and time-to-peak than TRH responses in each pair of data ( $P = 0.025$ ,  $n = 6$ , Wilcoxon matched-pairs signed-rank test). The maximum amplitude of the current was 200 nA for the GnRH response and 410 nA for the TRH response (both at 1  $\mu$ M) in the oocyte shown in Fig. 1. The mean values of the maximum amplitude for membrane current induced by GnRH and TRH (1  $\mu$ M) were  $187 \pm 82$  nA and  $272 \pm 156$  nA ( $n = 6$ ), respectively.

To avoid desensitization, the oocytes were usually exposed to peptide for short periods before being washed out. In oocytes continuously exposed to GnRH or TRH, however, the response lasted for longer periods (longer than 30 min). Oocytes became refractory to repeated applications of GnRH, as each successive current response showed longer latencies and smaller peak amplitudes ( $n = 5$ ). Similar results were found for TRH.

### Dose Dependence of the GnRH Response

The size and frequency of the current response induced by GnRH was dependent on the applied concentration of the peptide as shown in Fig. 2, where all traces were obtained from the same oocyte. At the concentration of 1 nM GnRH, individual low frequency inward current fluctuations could be observed. As the concentration of

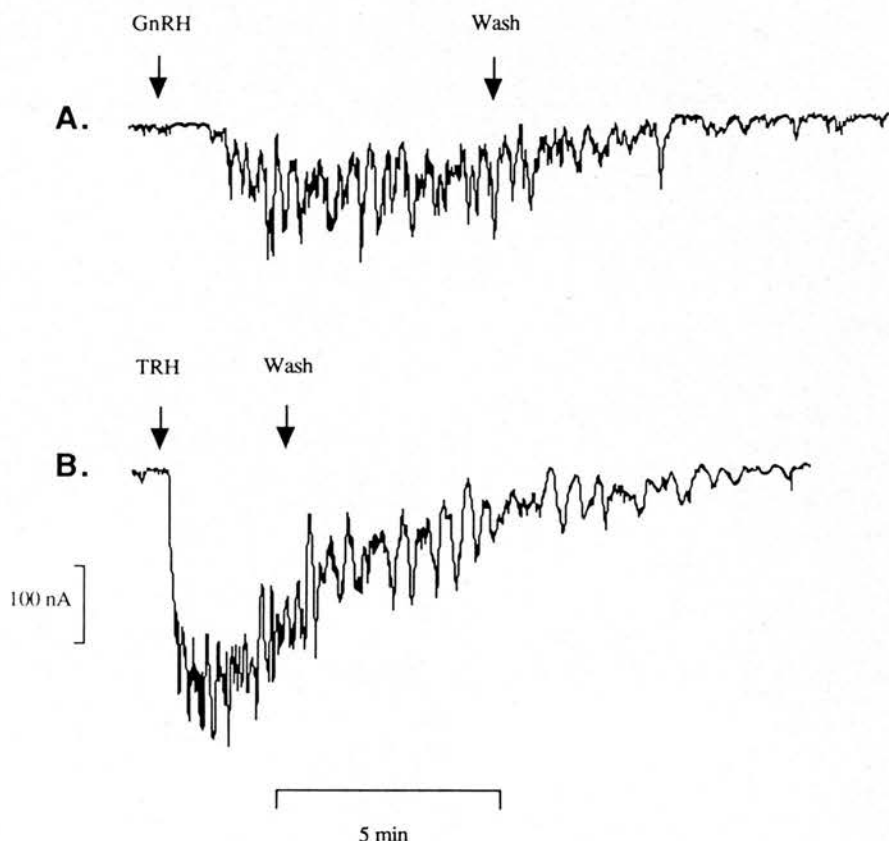
GnRH was increased, the current fluctuations became larger and more frequent, resulting in a sustained inward current with superimposed current fluctuations. Random application of the different concentrations of GnRH were tried, and the dose dependence of the response was assured qualitatively. An agonist of GnRH receptors (buserelin) was more potent in evoking current responses than GnRH at the same concentration (not illustrated). Selected oocytes ( $n = 5$ ) which showed small responses to GnRH were tested with GnRH agonist at the same concentration (1  $\mu$ M). The maximum current amplitude was  $16 \pm 12$  nA for GnRH and  $228 \pm 101$  nA for GnRH agonist ( $P = 0.025$ ,  $n = 5$ , Wilcoxon matched-pairs signed-rank test) indicating that GnRH agonist was much more potent than GnRH in inducing current responses.

### Inhibition of the GnRH Response by Cl<sup>-</sup> Channel Blockers

The current response evoked by GnRH was inhibited by the application of Cl<sup>-</sup> channel blockers such as 9-anthracene carboxylic acid (9-AC), diphenylamine-2-carboxylic acid (DPC), and 4-4'-diisothiocyanastilbene-2-2'-disulfonic acid (DIDS). An example of the blockade of the GnRH response by 9-AC, known to block Cl<sup>-</sup> channels in renal epithelia (15) and in *Xenopus* oocytes (16), is illustrated in Fig. 3. When GnRH (0.1  $\mu$ M) was applied to the oocyte a typical response was evoked, which was inhibited by the application of 9-AC (1 mM) in the continued presence of 0.1  $\mu$ M GnRH. When the inhibitor 9-AC was washed out with solution containing 0.1  $\mu$ M GnRH, the oscillating current response reappeared. DPC, another Cl<sup>-</sup> channel blocker described to be effective in epithelia (17), partially inhibited the GnRH response at a concentration of 0.1  $\mu$ M, and a complete blockade was obtained with 0.1 mM. DIDS (a classical Cl<sup>-</sup> channel blocker) abolished the GnRH response at the concentration of 0.1 mM (not shown). Current responses evoked by buserelin were similarly inhibited by these Cl<sup>-</sup> channel blockers. The results suggest that current responses induced by GnRH or GnRH agonist are dependent on the activity of the Cl<sup>-</sup> channels.

### Reversal Potential of the GnRH Response

The type of ions responsible for the GnRH response can be identified by measuring the reversal potential of the response. Since currents evoked by GnRH fluctuate and vary with time, two different methods were used to estimate the reversal potential. Firstly, the reversal potential of the current fluctuations was estimated by changing the holding potential as described by Gillo *et al.* (18) and by Boton *et al.* (16). The maximum amplitude of the current fluctuations was measured at different membrane potentials held for 1–1.5 min in oocytes which were continuously exposed to GnRH, and the maximum current amplitude was plotted against the membrane potential (Fig. 4). During these measurements, the oocytes response to the peptide did not



**Fig. 1.** Membrane Currents Induced by the Application of Peptides in an mRNA-Injected *Xenopus* Oocyte

The oocyte was injected with approximately 50 ng poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands and incubated for 6 days. Whole-cell current records were obtained using two-electrode voltage-clamp techniques. The holding potential was  $-60$  mV for both traces. A, Current fluctuations superimposed on a sustained current evoked by the application of  $1 \mu\text{M}$  GnRH. B, Current response induced by the application of  $1 \mu\text{M}$  TRH from the same oocyte shown in (A). The point of application of peptide is indicated by arrows, washout with standard solution followed. Peptides were introduced to and removed from the experimental chamber by perfusion. Current and time calibrations apply to both traces. Inward currents are shown as downward deflections in all figures.

desensitize. The I-V relationship was plotted and fitted using regression analysis, and the reversal potential was estimated from the point where the fitted line crossed the axis of the membrane potential  $V_m$  (the zero-current potential). The correlation coefficient of the fitted solid line shown in Fig. 4 was 0.987, and the reversal potential for the current was  $-26.3$  mV. The mean estimated reversal potential ( $n = 5$ ) for the current induced by busarelin or GnRH was  $-24.5 \pm 3.2$  mV. The correlation coefficient of fitted lines in these five examples was higher than 0.95, indicating the consistency of this method for estimating the reversal potential. The second method used for estimating the reversal potential was the ramp method (16, 18–20). Eight tenths to 1.0 sec ramp-command voltages with slopes of 100–150 mV/sec were used in the present study. Similar reversal potentials of approximately  $-25$  mV were obtained from this method ( $n = 3$ ).

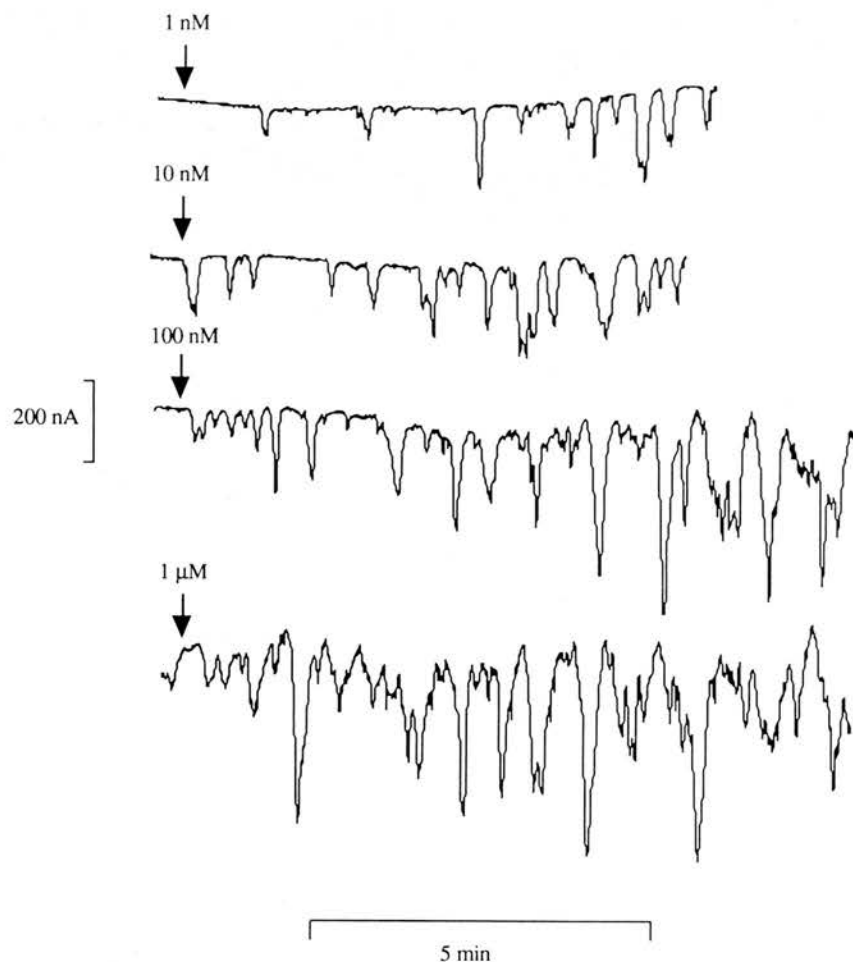
Dependence of the GnRH-induced current on Cl<sup>-</sup> ions was also demonstrated by changing the external concentration of Cl<sup>-</sup>. The concentration of Cl<sup>-</sup> was reduced to half (59.8 mM) by substituting the imper-

meant anion methanesulphonate for chloride. The linear portion of the I-V relationship in half Cl<sup>-</sup> was plotted against the holding potential (●, Fig. 4). The fitted dashed line ( $r = 0.998$ ) showed a parallel shift and the calculated reversal potential was  $-14.4$  mV. The mean reversal potentials for currents induced by GnRH recorded from the same oocytes in normal and half Cl<sup>-</sup> solutions were  $-25.2 \pm 3.0$  mV and  $-11.0 \pm 3.0$  mV ( $n = 3$ ), respectively. Therefore the average depolarizing shift of the reversal potential by halving the concentration of Cl<sup>-</sup> was 14.2 mV. This value is in good agreement with the expected value of 17.5 mV given by the Nernst equation  $E_{\text{Cl}^-} = RT/F \log_{10} [\text{Cl}^-]_i/[\text{Cl}^-]_o$  or  $E_{\text{Cl}^-} = 58.17 \log_{10} [\text{Cl}^-]_i/[\text{Cl}^-]_o$  at 20°C;  $E_{\text{Cl}^-}$  is the reversal potential for Cl<sup>-</sup> and the constants R, T and F have conventional meanings.

#### Dependence of the GnRH Response on Ca<sup>2+</sup>

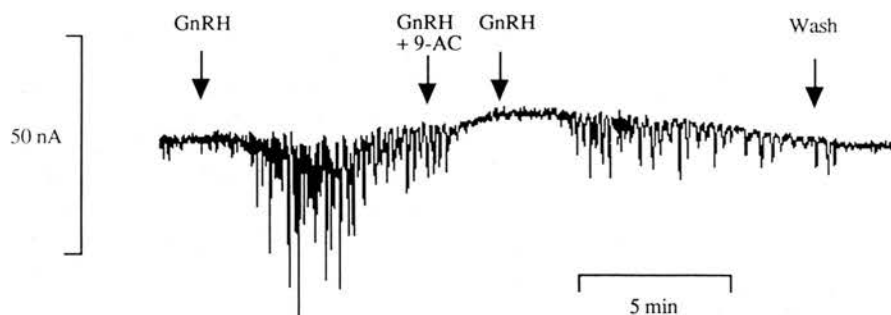
Since other exogenous receptors expressed in *Xenopus* oocytes have been shown to produce current re-





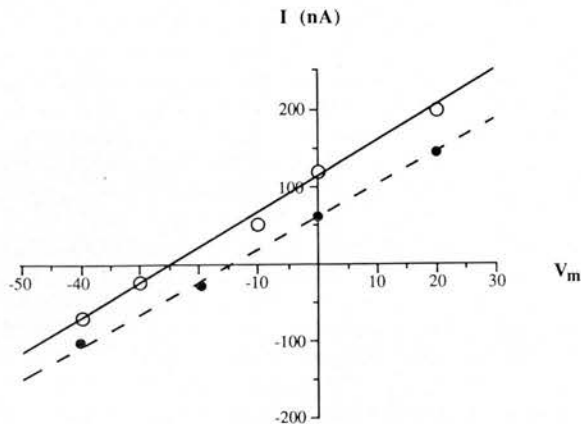
**Fig. 2.** Dose Dependence of the GnRH Response

Recordings made in *Xenopus* oocytes 4 days after injection of total poly(A)<sup>+</sup> mRNA isolated from rat anterior pituitary glands. The oocyte was clamped at  $-60$  mV. Serial recordings were obtained by increasing the concentrations of GnRH in the bathing medium by perfusion in steps as indicated by arrows. Current and time calibrations are the same for all traces.



**Fig. 3.** Inhibition of the GnRH response by a  $\text{Cl}^-$  Channel Blocker

The current response was evoked by the application of  $0.1 \mu\text{M}$  GnRH and was reversibly blocked by  $1 \text{ mM}$  9-AC in an mRNA-injected oocyte. The recovered GnRH response disappeared when GnRH was washed out. Arrows indicate the point of chemical application or washing out with standard solution.



**Fig. 4.** Current-Voltage (*I*-*V*) Relation of the Response Induced by Buserelin (an Agonist of GnRH Receptors)

The observed maximum amplitude of current fluctuations induced by buserelin were plotted against the holding potential. ○, ●, obtained from the same oocyte, were recorded in normal [Cl<sup>-</sup>]<sub>o</sub> (119.6 mM) and in half [Cl<sup>-</sup>]<sub>o</sub> (59.8 mM) solutions, respectively. The lines were fitted by linear regression analysis.

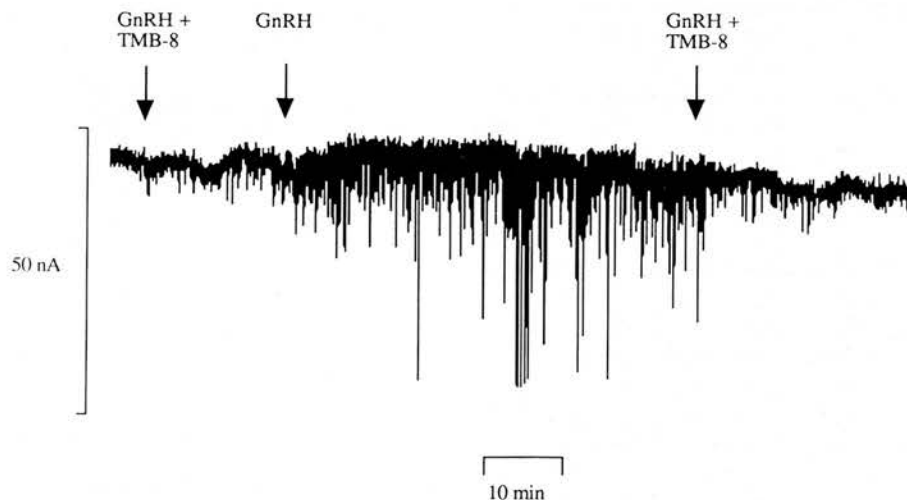
sponses dependent on the activity of Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels (7, 8), the dependence of the Cl<sup>-</sup> channel activity on internal Ca<sup>2+</sup> was examined for the GnRH response. The GnRH response was inhibited by 8-(*N*, *N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), a blocker of intracellular Ca<sup>2+</sup> release (21) (*n* = 4). An example is shown in Fig. 5. TMB-8 was applied to an mRNA-injected oocyte at the concentration of 0.2 mM with 1 μM GnRH; no significant response occurred in membrane current. TMB-8 was then removed from the bathing solution, and the GnRH response was elicited. The response was suppressed when TMB-8 was reintroduced.

Chlordiazepoxide, which has been shown to interfere

with GnRH-stimulated LH release from pituitary cell cultures by inhibiting calmodulin activity (22), was also tested on mRNA-injected oocytes. The GnRH response was inhibited by the addition of 0.2–1 μM chlordiazepoxide (*n* = 4) (not shown).

## DISCUSSION

The results presented above indicate that the responses to GnRH or buserelin measured in *Xenopus* oocytes injected with rat pituitary mRNA were mainly dependent on the activity of Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels. The GnRH response was inhibited by the Cl<sup>-</sup> channel blockers 9-AC, DPC, and DIDS. The oscillatory nature of the GnRH response resembles the current fluctuations induced by GnRH in rat gonadotropes (23). Increasing the GnRH concentrations from 1 nM to 1 μM increased the amplitude of these current fluctuations in a dose-dependent manner. In perfused sheep pituitary cells and isolated rat pituitary cells doses of GnRH in the range 10<sup>-11</sup>–10<sup>-6</sup> M gave rise to release of LH and FSH (Taylor, PL, and K. A. Eidne, unpublished observations), so the doses used in this study are comparable with those used in other experimental situations. The reversal potential for the GnRH response was approximately -25 mV, and this value agrees well with the reported reversal potential for Cl<sup>-</sup> in *Xenopus* oocytes (19, 24–26). Further evidence for the dependency of the GnRH response on Cl<sup>-</sup> was provided by the observed shift in the reversal potential with the change in concentration of external Cl<sup>-</sup>. The depolarizing shift of the reversal potential produced by halving the external Cl<sup>-</sup> concentration was 14.2 mV, similar to the value of 17.5 mV predicted by the Nernst equation for a Cl<sup>-</sup> electrode.



**Fig. 5.** Suppression of the GnRH Response by a Blocker of Intracellular Ca<sup>2+</sup> Release, TMB-8

GnRH was firstly applied to an oocyte with TMB-8 (0.2 mM). The GnRH response was elicited when TMB-8 was removed from the bathing solution and inhibited again when it was reintroduced. The points of chemical applications are indicated by arrows.

The activity of the  $\text{Cl}^-$  channel responsible for the GnRH response was dependent on internal  $\text{Ca}^{2+}$ . TMB-8, a blocker of intracellular  $\text{Ca}^{2+}$  release (21), inhibited the GnRH response. Chlordiazepoxide has been reported to inhibit GnRH-stimulated LH release by binding to calmodulin in rat pituitary cell cultures (22). Also, chlordiazepoxide has been shown to inhibit the TRH response induced in *Xenopus* oocytes injected with RNA derived from GH<sub>3</sub> cells of rat pituitary tumor origin (11). The present data show that the GnRH response in mRNA-injected *Xenopus* oocytes was also inhibited by chlordiazepoxide suggesting that these responses may be dependent on calmodulin which controls intracellular  $\text{Ca}^{2+}$ . It is therefore likely that the GnRH response is dependent on the mobilization of intracellular  $\text{Ca}^{2+}$  for the activation of  $\text{Cl}^-$  channels. The direction of the current carried by  $\text{Cl}^-$  ( $I_{\text{Cl}^-}$ ) in response to GnRH or busserelin can be described by the equation  $I_{\text{Cl}^-} = g_{\text{Cl}^-}(E - E_{\text{Cl}^-})$  where  $E$  and  $E_{\text{Cl}^-}$  indicate the membrane potential and the reversal potential for  $\text{Cl}^-$ , respectively. Under voltage-clamp conditions when the holding potential is set at  $-60$  mV (close to the resting potential), the driving force for  $\text{Cl}^-$ , i.e.  $(E - E_{\text{Cl}^-})$  is negative because the equilibrium potential for  $\text{Cl}^-$  is approximately  $-25$  mV, since the  $\text{Cl}^-$  conductance ( $g_{\text{Cl}^-}$ ) is positive  $I_{\text{Cl}^-}$  must therefore be negative. Negative (or inward) current means an efflux of  $\text{Cl}^-$ . This inward current would explain the depolarizing membrane-potential change observed with intracellular recording techniques in our previous paper (14). As the resting potential of oocytes ( $\sim -60$  mV) is more negative than the equilibrium potential for  $\text{Cl}^-$  ( $\sim -25$  mV), the oocyte membrane potential shifts toward this equilibrium potential, resulting in a membrane depolarization as the membrane becomes permeable to  $\text{Cl}^-$  in response to GnRH or busserelin.

So far, exogenous receptors expressed in *Xenopus* oocytes for acetylcholine, serotonin, TRH, and substance K produce current responses dependent on the activity of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (7, 8). Recently, the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance in *Xenopus* oocytes was further classified into two components, the fast, transient one, and the slow one (16). These two components have different  $\text{Ca}^{2+}$  dependence of activation and inactivation and different sensitivity to a  $\text{Cl}^-$  channel blocker. The slow component was less sensitive to 9-AC (the amplitude of slow current was only reduced by 59% by 2 mM 9-AC). The  $\text{Cl}^-$  conductance in response to GnRH in the present study resembles the slow component of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance in its time course but is more sensitive to 9-AC. We have noted that higher concentrations of 9-AC and DPC, potent  $\text{Cl}^-$  channel blockers in epithelia (15, 17), were necessary to be effective in *Xenopus* oocytes. It is possible that ion channels in undifferentiated cells such as *Xenopus* oocytes are less sensitive to these blockers than those in epithelia. Indeed, it has been shown that  $\text{Ca}^{2+}$  channels in mouse oocytes are less sensitive to a  $\text{Ca}^{2+}$  channel blocker, diltiazem, than in differentiated cells (27).

It is to be noted that the TRH response observed in

the present study was not preceded by the fast response. Similar findings have been reported by Meyerhof *et al.* (11) who used *Xenopus* oocytes injected with mRNA isolated from GH<sub>3</sub> cells of rat pituitary tumor origin. In contrast, Oron *et al.* (9, 10) always observed a TRH response which consisted of a transient, rapid response followed by a long-lasting current with superimposed current fluctuations in *Xenopus* oocytes injected with mRNA isolated from GH<sub>3</sub> cells. That the absence of the fast component was not due to limitations of the recording equipment in our experiments, was shown by recording the response to acetylcholine (18, 28) as shown in Fig. 6. These variations in the fast component of the TRH response may be due to the different source of mRNA which determines the density of receptors expressed on oocyte membranes.

The present study has shown that the rat anterior pituitary gland mRNA encoding the GnRH receptor can be translated in *Xenopus* oocytes. These receptors are able to couple up to G protein-linked second messenger systems present in *Xenopus* oocytes. The mechanism of this response depends on the activity of chloride channels which are endogenous in the oocytes (7, 8). Since *Xenopus* oocytes have been successfully used as part of a cloning strategy to isolate several receptors (12, 13), this system could also be used to isolate a cDNA clone encoding the GnRH receptor.

## MATERIALS AND METHODS

### Preparation of Rat Anterior Pituitary RNA

Total RNA was extracted from anterior pituitary glands of adult female rats as previously described (14). Oligo(dT) affinity

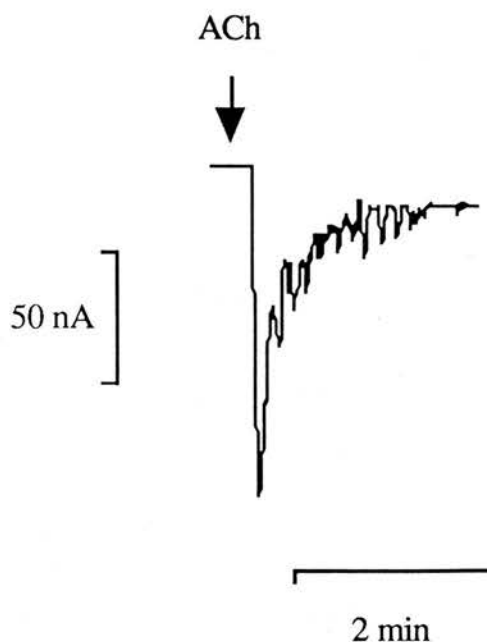


Fig. 6. A Current Response to Acetylcholine (ACh) Recorded in a Control Oocyte

Uninjected *Xenopus* oocyte, exposed to  $10 \mu\text{M}$  ACh by perfusion, showing membrane current consisting of a fast response followed by slow current fluctuations.

chromatography was used to obtain a poly(A)<sup>+</sup>-enriched mRNA fraction (29).

### Microinjection of mRNA into *Xenopus* Oocytes

Oocytes were collected from adult female *Xenopus laevis* by dissection from their surrounding tissues followed by defolliculation with collagenase (6 mg/ml for 1–3 h at room temperature) (25), and were kept overnight at 19°C in modified Barth's solution supplemented with penicillin (10 IU/ml) and streptomycin (10 µg/ml). Messenger RNA was dissolved in deionized water at a concentration of approximately 1 mg/ml, and each oocyte was injected with 50 nl into the vegetal hemisphere close to the equatorial line. Untreated oocytes and oocytes injected with 50 nl deionized water were used for control.

### Electrophysiology

Whole-cell current measurements in *Xenopus* oocytes were carried out using a conventional two-electrode voltage-clamp amplifier (25) with glass microelectrodes filled with 3 M KCl. DC resistances of microelectrodes were 3–10 MΩ and 0.8–1.5 MΩ for voltage-recording and current-injection electrodes, respectively. The resting potential measured with a single voltage-recording electrode ranged between -47 and -76 mV. The average resting potential was  $-61.8 \pm 8.2$  mV ( $n = 20$ ). Oocytes were routinely voltage-clamped at -60 mV. Membrane currents were processed by a digital audioprocessor (SONY PCM-701ES, Japan; sampling frequency 44.1 kHz) modified to accept down to DC (30) and stored on video tapes with a conventional video cassette recorder (Panasonic NV-G12B, Japan). The stored signal was digitized at a sampling frequency of 2 Hz with an interface (Analog Highway; 3D Digital Design and Development Ltd., London, UK) and fed to a computer (Macintosh Plus, Cupertino, CA) for analysis with software developed by one of us (Taylor, P. L.). All experiments were performed at room temperature (19–23°C). Measurements were carried out 1–6 days after injection of mRNA.

### Solutions

The ionic composition of the standard solution used for electrophysiology was: 115 mM NaCl, 1 mM KCl, 1.8 mM CaCl<sub>2</sub>, and 10 mM HEPES (pH 7.4). The impermeant anion methanesulfonate was substituted for chloride when necessary. GnRH and buserelin ([D-Ser(But)<sup>6</sup>, Pro<sup>9</sup>-NEt<sup>1</sup>]GnRH) were obtained from Farbwerke Hoechst (Frankfurt, FRG). TRH was purchased from Cambridge Research Biochemicals (Cambridge, UK). Cl<sup>-</sup> channel blockers used in the present study were 9-AC (Aldrich, Milwaukee, WI), DIDS (Fluka, Buchs, Switzerland), and DPC (Fluka). 9-AC and DIDS were firstly dissolved in dimethyl sulfoxide and DPC was dissolved in hot ethanol. The final concentration of dimethyl sulfoxide and ethanol in the bathing medium were 0.1 and 0.01% (vol/vol), respectively. Vehicle alone at these concentrations had no effect on responding oocytes. Chlordiazepoxide was obtained from Sigma (St. Louis, MO) and TMB-8 was from Calbiochem (La Jolla, CA). The pH of all solutions was adjusted to 7.4. Peptides and blockers were introduced to and removed from the bathing medium by perfusion.

### Statistical Tests

The Wilcoxon matched-pairs signed-rank test was used for statistical analysis of pairs of data considering the direction and magnitude of change in the values (31).

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